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Determining the contribution of Mx1 in the antiviral immune cell compartment

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Summary

Influenza or the flu, which is caused by infection with an influenza virus, is characterized by symptoms such as fever, cough, headache, muscle and joint pain, severe malaise, a sore throat and a runny nose. Most people recover within one week after onset of symptoms. However, influenza viruses cause three to five million severe cases of illness and approximately 250 000 to 500 000 fatalities worldwide every year, particularly in children, elderly and people with underlying malignancies or other infections[1]. Influenza virus is easily transmitted by both direct contact and through the air. The latter is particularly of importance in crowded areas, as sneezing or coughing produces small virus-containing droplets which spread easily to nearby persons who breathe in these droplets. In the respiratory tract, influenza viruses infect airway epithelial cells. After entry in these cells, the viral genetic material, in the form of viral ribonucleoproteins (vRNPs), is released into the cytoplasm and subsequently enters the nucleus. In the nucleus, transcription and replication occurs. The former leads to production of viral mRNA, which is transported to the cytosol for translation into viral proteins. Some of these newly produced proteins shuttle back to the nucleus where they initiate the replication of the viral genome. The newly produced viral RNA molecules form new vRNPs which leave the nucleus and associate with structural viral proteins to form new virus particles. After budding, released viruses can infect neighboring cells and the cycle starts over again[2].

More than 50 years ago Lindenmann *et al.* discovered a gene which conferred resistance against influenza A virus (FLUAV) infection in mice, and which is now known as *Mx1*[3-5]. The term Mx signifies myxovirus-resistance, because mice that can express a functional form of this protein are resistant to infection with influenza A virus and other myxoviruses[4,5]. Quite some years later, a human ortholog of murine Mx1 was isolated and named MxA[6]. Hitherto, several Mx orthologs have been described in practically all vertebrates, and they confer resistance against a whole array of viruses such as *Orthomyxoviridae*, *Rhabdoviridae*, and *Bunyaviridae* (reviewed by Verhelst *et al.*[7]). The exact mechanism by which murine Mx1 exerts viral inhibition is largely unknown. Our lab has contributed to the elucidation of the influenza-specific antiviral mechanism by showing that Mx1 interacts with the nucleoprotein (NP) and polymerase basic 2 (PB2) protein of influenza A viruses and, that in the presence of mouse Mx1 the interaction between the NP and PB2 molecules is abolished[8]. We also found evidence that Mx1 might actively disrupt existing influenza A vRNPs[9]. Being a type I interferon-inducible protein, Mx1 is thought to primarily exert an innate antiviral effect by reducing FLUAV early after infection, and preventing viral spread through the airways. It is not known whether Mx1 could also fulfill a role in the antiviral immune cell compartment after a FLUAV infection. The induction by type I IFNs of an

antiviral state in antigen presenting cells and in memory T cells has been reported to directly affect the immune response against a primary and secondary influenza virus infection, respectively[10,11]. In addition, human DCs rapidly upregulate MxA and thus, at least in vitro, become resistant to the virus and can sustain antigen presentation[12]. Most *in vivo* studies which examine the FLUAV-induced immune response are performed using mouse strains which do not possess a functional *Mx1* locus[13]. Based on such models, it has been reported that certain immune cell types, *i.e.* $CD103^+$ dendritic cells and lung resident memory $CD8^+$ T cells, are protected against FLUAV infection due to an interferon-induced antiviral state[10,11]. This was the reason for us to hypothesize that Mx1 could also play a role in the formation of this antiviral state.

To address this question, we set up an infection model wherein we made use of bone marrow chimeric mice. Since most immune cell types have a hematopoietic origin, bone marrow transfer from mice with a functional *Mx1* locus (B6.A2G $Mx1^{+/+}$) to mice without a functional *Mx1* locus (B6.A2G $Mx1^{-/-}$), and *vice versa*, allowed us to study the possible function of Mx1 in bone marrow-derived cell types. The bone marrow chimeric mice were infected with a high dose of FLUAV. Multiple parameters were examined (body weight, lung viral titers, viral mRNA and protein levels), and it was apparent that Mx1 expression in bone marrow-derived cell types was not the main factor determining resistance against FLUAV infection. The driving force in resistance against FLUAV infection was whether or not Mx1 is expressed in stromal cells. This difference between $Mx1^{-/-}$ and $Mx1^{+/+}$ recipient mice was also noticeable in the evolution of the levels of eosinophils, monocyte-derived dendritic cells, and alveolar macrophages in the lung.

Results obtained with the FLUAV infection model were not conclusive to address the hypothesis that Mx1 could play a role in bone marrow-derived cell types after viral infection. Therefore, we tried a second Orthomyxovirus infection model. Thogoto virus (THOV) is a tick-borne virus which, like FLUAV, belongs to the family of Orthomyxoviruses. Importantly, small rodents are natural hosts for this virus, and THOV is also sensitive to inhibition by Mx1[14,15]. Bone marrow chimeric mice were infected with a high dose of THOV. Again, multiple parameters were examined (body weight, liver viral titers, viral protein levels, liver pathology). Like in the FLUAV infection model, the main determinant for resistance against infection is Mx1 expression in the stromal cells. However, irradiated $Mx1^{-/-}$ recipient mice that had received $Mx1^{+/+}$ bone marrow cells, displayed reduced morbidity from the THOV infection compared with $Mx1^{-/-}$ recipients which received $Mx1^{-/-}$ bone marrow cells as evidenced by the reduced weight loss and liver pathology which was observed for this group of mice. This observation suggests that *Mx1* can play an important

role in immune cell types after viral infection, although the importance of this role is largely dependent on the infecting virus.

We also addressed the possible contribution of Mx1 in context of a vectored influenza A NP vaccine antigen. Recently, Altenburg *et al.* examined whether recombinant modified vaccinia Ankara (rMVA) vaccines which expressed mutated forms of NP would elicit a stronger antigen-specific immune response than rMVA vaccines expressing the wild type (WT) form of NP (rMVA NPwt). The introduced mutations were intended to enhance cytosolic retention or degradation of the NP molecules. For this, they either mutated the nuclear localization signal (NLS) (rMVA-NPmut), deleted the NLS (rMVA-NPΔNLS) or fused ubiquitin to NP (rMVA-UbqNP). *In vitro*, these mutated NP constructs outperformed rMVA-NPwt in activating NP-specific T cells. However, immunization of C57BL/6 mice with the mutant rMVA-NP constructs did not result in significantly higher NP-specific $CD8^+$ T cell responses or protection against influenza A virus challenge than the rMVA-NPwt construct[16]. We reasoned that this might be because the required threshold of processed NP antigen for a robust $CD8^+$ T cell response may be readily reached by the WT NP and thus difficult to improve further by NP variant constructs. Therefore, we speculated that mice which do express a functional Mx1 protein, as opposed to C57BL/6 mice, would be a better suited model to test these different rMVA-NP constructs. Mx1, which has been shown to interact with NP[8], could be the additional restriction factor needed to demonstrate the advantage of these mutated NP constructs.

We vaccinated B6.A2G $Mx1^{-/-}$ and B6.A2G $Mx1^{+/+}$ mice with the different rMVA-NP constructs. One week after the second immunization the NP-specific $CD8^+$ T cell response was examined by intracellular cytokine staining (ICS) and enzyme-linked immunospot (ELISPOT) assay using blood and spleen. Both ICS and ELISPOT data showed no significant differences between the mutated and the WT NP constructs in B6.A2G $Mx1^{+/+}$ mice. However, ELISPOT data showed a trend that rMVA-NPmut and rMVA-NPΔNLS constructs elicit a stronger $CD8^+$ T cell response than the rMVA-NPwt construct. To further substantiate the theory that Mx1 might act as a determinant for the induction of NP-specific cellular responses, additional experiments will have to be performed.

The results obtained with the THOV infection model show clearly that *Mx1* plays a role of significance in immune cells upon viral infection. Consequently, we could draw two major conclusions from this thesis. First, when studying an infection model it is imperative to use a well suited combination of host and virus. Since THOV is a natural pathogen of small rodents, it is ideal to use in a mouse model. Second, additional to the choice of a suitable host-virus combination, we can also conclude that the tropism of the chosen virus is of great

importance. When investigating the antiviral role of a protein in a certain cell type, it is essential that the chosen virus infects this cell type. THOV infects myeloid CD11b^{+} cells[17], and was consequently very well suited for our experiments.

Samenvatting

Influenza of de griep is een ziekte die wordt veroorzaakt door een infectie met het influenza virus en ze wordt gekenmerkt door symptomen als koorts, hoesten, hoofdpijn, pijnlijke spieren en gewrichten, algehele malaise, keelpijn en een loopneus. Meestal is men binnen een week na de eerste symptomen hersteld. Toch veroorzaken influenza virussen jaarlijks drie tot vijf miljoen ernstige gevallen van ziekte en leiden ze tot ongeveer 250 000 tot 500 000 sterfgevallen wereldwijd. Vooral kinderen, ouderen, en personen met onderliggende aandoeningen of andere infecties zijn zeer gevoelig voor infecties met het influenza virus[1]. Het influenza virus wordt makkelijk overgedragen van mens tot mens, zowel via rechtstreeks contact als via de lucht. Dit laatste is vooral belangrijk in drukke omgevingen, want door niezen en hoesten worden er kleine druppeltjes gevormd die het virus bevatten. Deze druppeltjes worden makkelijk verspreid naar personen in de nabije omgeving, die deze dan op hun beurt inademen. Hierna kunnen de influenza virussen epitheelcellen infecteren in de luchtwegen. Wanneer het virus de cellen is binnengedrongen, zal het zijn genetisch materiaal vrijstellen in het cytoplasma onder de vorm van virale ribonucleoproteïnen (vRNP's) die vervolgens de celkern binnengaan. In de celkern vindt de transcriptie en de replicatie plaats. Transcriptie leidt tot de productie van mRNA en dit mRNA wordt vervolgens naar het cytosol gebracht waar het vertaald wordt tot virale eiwitten. Sommige van deze virale eiwitten gaan naar de celkern waar ze de replicatie van het virale genoom opstarten. Replicatie leidt tot de productie van nieuwe virale RNA moleculen die aanleiding geven tot de vorming van nieuwe vRNP's. Deze vRNP's verlaten vervolgens de celkern en vormen nieuwe viruspartikels samen met structurele virale eiwitten. De nieuwe virussen komen vrij uit de geïnfecteerde cel en kunnen de naburige cellen infecteren, waardoor de hele cyclus van voor af aan begint[2].

Meer dan 50 jaar geleden ontdekten Lindenmann *et al.* een gen dat ervoor zorgt dat muizen resistent zijn tegen infectie door het influenza A virus (FLUAV). Dit gen werd *Mx1* genoemd[3-5], wat staat voor *myxovirus resistance 1*. Deze naam werd gegeven omdat muizen die een functionele vorm van het eiwit aanmaken resistent zijn tegen infecties door FLUAV en andere myxovirussen[4,5]. Een tijd later werd een humane ortholoog van muis *Mx1* geïsoleerd, en dit gen werd *MxA* genoemd[6]. Tot nu toe zijn er verschillende *Mx* orthologen beschreven in zo goed als alle vertebraten. Deze orthologen verlenen resistentie tegen een hele resem aan virussen zoals *Orthomyxoviridae*, *Rhabdoviridae*, en *Bunyaviridae*[7]. Het exacte mechanisme waarmee muis Mx1 virussen onderdrukt, is nog steeds grotendeels onbekend. Onze onderzoeksgroep heeft bijgedragen aan de ontrafeling van het specifieke anti-influenza mechanisme door aan te tonen dat Mx1 een interactie aangaat met het nucleoproteïne (NP) en het polymerase basisch (PB2) eiwit van FLUAV, en dat in de

aanwezigheid van muis Mx1 de interactie tussen deze twee eiwitten wordt verhinderd[8]. We hebben ook bewijzen gevonden dat het Mx1 eiwit reeds gevormde influenza A vRNP's actief zou kunnen verbreken[9]. Aangezien Mx1 een type I interferon-induceerbaar eiwit is, vermoedt men dat het voornamelijk vroeg na de infectie FLUAV onderdrukt en het virus op deze manier verhindert zich in de luchtwegen te verspreiden. Het is niet bekend of Mx1 een rol kan spelen in het immuun cel compartiment na een infectie door FLUAV. De inductie van een antivirale status, door type I interferon, in antigenpresenterende cellen of geheugen T cellen heeft een direct effect op het immuunsysteem tegen respectievelijk primaire of secundaire influenza virus infecties[10,11]. Daarenboven kunnen humane dendritische cellen MxA snel opreguleren en waardoor ze, tenminste *in vitro*, resistent worden tegen het virus en de antigenpresentatie in stand kunnen houden[12]. De meeste studies die het immuunantwoord opgewekt door FLUAV bestuderen maken gebruik van muisstammen die geen functionele vorm van Mx1 kunnen produceren[13]. Op basis van deze studies wordt beweerd dat bepaalde types immuuncellen zoals $CD103^+$ dendritische cellen en long residente geheugen $CD8^+$ T cellen, beschermd zijn tegen FLUAV infecties doordat ze een interferon-geïnduceerde antivirale status aannemen[10,11]. Hierdoor is de hypothese ontstaan dat Mx1 ook een rol zou kunnen spelen in het vormen van de antivirale status in deze cellen.

Om deze hypothese te onderzoeken, hebben we een infectiemodel opgezet waarin we gebruik maakten van beenmergchimeren. Aangezien de meerderheid van de immuunceltypes voortkomen uit het beenmerg, zal een beenmergtransplantatie van muizen met een functioneel *Mx1* gen (B6.A2G $Mx1^{+/+}$) naar muizen zonder een functioneel *Mx1* gen (B6.A2G $Mx1^{-/-}$) en *vice versa*, ons toelaten om de eventuele functie van Mx1 te bestuderen in celtypes die afkomstig zijn uit het beenmerg. De chimere muizen werden geïnfecteerd met een hoge dosis FLUAV en meerdere parameters werden onderzocht (lichaamsgewicht, virale titers in de long, virale mRNA- en eiwitniveaus in de long). Hieruit konden we afleiden dat Mx1-expressie in celtypes afkomstig uit het beenmerg niet de voornaamste factor was die de resistentie tegen een FLUAV infectie bepaalde. De drijvende kracht achter de resistentie tegen FLUAV infecties was de aanwezigheid van Mx1 in stromale cellen. Dit verschil tussen $Mx1^{-/-}$ en $Mx1^{+/+}$ recipiënt muizen was ook merkbaar bij de verandering van de hoeveelheden eosinofielen, monocytafgeleide dendritische cellen en alveolaire macrofagen in de long.

De reeds bekomen resultaten boden echter geen sluitend bewijs om onze hypothese te bevestigen. Daarom hebben we een tweede infectiemodel opgezet met het Thogoto virus (THOV), een virus dat wordt overgedragen door teken. THOV is, net als FLUAV, een lid van de familie van de Orthomyxovirussen. Bovendien zijn kleine knaagdieren zoals muizen en

ratten de natuurlijke gastheren van dit virus en is THOV ook gevoelig voor inhibitie door Mx1[14,15]. Chimere muizen werden geïnfecteerd met een hoge dosis THOV en wederom werden meerdere parameters onderzocht (lichaamsgewicht, virale titers in de lever, virale eiwitniveaus in de lever, leverpathologie). Net als bij het FLUAV infectiemodel is Mx1-expressie in de stromale cellen de belangrijkste determinant voor resistentie tegen infectie. Toch vertoonden bestraalde $Mx1^{-/-}$ recipiënt muizen die $Mx1^{+/+}$ beenmergcellen ontvingen een gereduceerde morbiditeit na THOV infectie in vergelijking met $Mx1^{-/-}$ recipiënt muizen die $Mx1^{-/-}$ beenmergcellen ontvingen. Dit uitte zich in minder verlies van lichaamsgewicht en minder ernstige leverpathologie bij deze groep muizen. Deze observatie toont aan dat *Mx1* een belangrijke rol kan spelen in immuunceltypes na een virale infectie, hoewel het belang van deze rol sterk afhankelijk is van het infecterende virus.

Als tweede luik van deze thesis hebben we onderzocht wat de bijdrage van Mx1 kan zijn in de context van een vaccinatie met een influenza A NP antigen dat door middel van een virale vector wordt toegediend. Altenburg *et al.* hebben recentelijk onderzocht of recombinante gemodificeerde vaccinia Ankara (rMVA) vaccins die gemuteerde vormen van het FLUAV NP eiwit aanmaken, een sterkere antigenspecifieke immuunrespons zouden opwekken dan rMVA vaccins die de wild type (WT) vorm van NP aanmaken (rMVA-NPwt). De aangebrachte mutaties waren bedoeld om de NP moleculen in het cytosol te houden of om hun afbraak tot peptiden te verbeteren. Hiervoor werd het nucleair lokalisatiesignaal (NLS) van NP gemuteerd (rMVA-NPmut) of volledig verwijderd (rMVA-NPΔNLS), of werd ubiquitine aan NP gekoppeld (rMVA-UbqNP). De gemuteerde NP constructen konden *in vitro* NP-specifieke T cellen beter activeren dan het rMVA-NPwt construct. Dit effect kon echter niet doorgetrokken worden naar een *in vivo* situatie, want vaccinatie van C57BL/6 muizen met de verschillende rMVA-NP constructen leidde niet tot een significant verhoogde NP-specifieke $CD8^+$ T cel respons of een betere bescherming tegen een FLUAV infectie dan het geval was voor het rMVA-NPwt construct[16]. Dit resultaat komt waarschijnlijk voort uit het feit dat het rMVA-NPwt construct gemakkelijk de vereiste drempelwaarde voor NP-antigen kan bereiken en dus moeilijk verbeterd kan worden door deze variante NP constructen. Deze drempelwaarde voor NP-antigen moet bereikt worden om een robuuste $CD8^+$ T cel respons te kunnen ontwikkelen. Daarom vermoedden wij dat muizen die een functioneel Mx1 eiwit aanmaken een beter model zouden zijn dan C57BL/6 muizen, die geen functioneel Mx1 eiwit aanmaken[13], om deze verschillende rMVA-NP constructen te testen. Mx1, waarvan is aangetoond dat het een interactie aangaat met NP[8], kan optreden als de extra restrictiefactor die nodig is om het voordeel van de gemuteerde NP constructen aan te tonen.

Daarom werden B6.A2G $Mx1^{-/-}$ en B6.A2G $Mx1^{+/+}$ muizen gevaccineerd met de verschillende rMVA-NP constructen. Een week na de tweede vaccinatie werd de NP-specifieke $CD8^+$ T cel respons onderzocht in bloed en milt door middel van *intracellular cytokine staining* (ICS) en *enzyme-linked immunospot assay* (ELISPOT). Zowel de resultaten bekomen met ICS als met ELISPOT vertoonden geen significante verschillen tussen de gemuteerde constructen en het WT NP construct in B6.A2G $Mx1^{+/+}$ muizen. Desalniettemin vertoonden de ELISPOT resultaten wel de trend dat rMVA-NPmut en rMVA-NPΔNLS constructen een sterkere $CD8^+$ T cel respons opwekken dan het rMVA-NPwt construct. Maar om de theorie dat Mx1 een belangrijke factor zou kunnen zijn in de inductie van een NP-specifieke cellulaire respons na vaccinatie met gemuteerde NP constructen zijn er bijkomende experimenten nodig.

De resultaten die we bekomen hebben met het THOV infectiemodel tonen duidelijk aan dat Mx1 na virale infectie een belangrijke rol speelt in immuuncellen. Bijgevolg konden we twee grote conclusies trekken uit deze thesis. Ten eerste is het bij het bestuderen van een infectiemodel is het van groot belang om een gepaste combinatie van gastheer en virus te gebruiken. Aangezien THOV een natuurlijke pathogeen is voor kleine knaagdieren, is dit virus ideaal om te gebruiken in een muismodel. Ten tweede is het tropisme van het geselecteerde virus is ook zeer belangrijk. Bij het onderzoeken van de antivirale rol van een eiwit in een bepaald celtype is het essentieel dat het gebruikte virus dit celtype ook effectief kan infecteren. THOV infecteert myeloïde $CD11b^+$ cellen[17], en bleek dus uitermate geschikt voor deze experimenten.

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List of abbreviations

#	25HC	25-hydroxycholesterol
	3D	three-dimensional
A	ADCC	antibody-dependent cellular cytotoxicity
	AM	alveolar macrophage
	APC	antigen-presenting cell
	ASC	adaptor protein apoptosis-associated speck-like protein containing a CARD domain
B	BSE	bundle signaling element
	BST2	bone marrow stromal antigen 2
C	CBP	CREB-binding protein
	CCL	chemokine (C-C motif) ligand
	CCR	C-C chemokine receptor
	CD	cluster of differentiation
	CD62L	L-selectin
	cDC1	conventional dendritic cell type 1
	cDC2	conventional dendritic cell type 2
	CH25H	cholesterol 25-hydroxylase
	cRNA	complementary ribonucleic acid
	CREB	cAMP-response element-binding protein
	CRM1	chromosome region maintenance 1
	CTL	cytotoxic T lymphocyte
	CXCL	chemokine (C-X-C motif) ligand
D	DC	dendritic cell
	DDX	DExD-box
	DLN	draining lymph node
	dpi	days post infection
E	eIF4	eukaryotic translation initiation factor 4
	ELISPOT	enzyme-linked immunospot assay
	ER	endoplasmic reticulum

F	FAM3B	family with sequence similarity 3 member B
	FasL	Fas ligand
	FLUAV	influenza A virus
	FPPS	farnesyl diphosphate synthase
G	GED	GTPase effector domain
	GDP	guanosine diphosphate
	GP	glycoprotein
	GTP	guanosine triphosphate
H	H&E	hematoxylin and eosin
	HA	hemagglutinin
	HCV	hepatitis C virus
	HI	hemagglutination-inhibiting
	HIV-1	human immunodeficiency virus type 1
	Hsp90	heat shock protein 90
I	ICAM-1	intercellular adhesion molecule 1
	ICS	intracellular cytokine staining
	IFITM	interferon-induced transmembrane
	IFN	interferon
	Ig	immunoglobulin
	IL	interleukin
	IRF3	interferon regulatory factor 3
	ISG	interferon-stimulated gene
	iTreg	induced regulatory T cell
L	L4	loop 4
	LAIV	live-attenuated influenza vaccine
	LD_{50}	median lethal dose
	LFA-1	lymphocyte function-associated antigen 1
	LPM	large peritoneal macrophage
M	M	matrix protein
	M1	matrix protein 1
	M2	matrix protein 2
	maPR8	mouse-adapted influenza A/Puerto Rico/8/34
	MCP	monocyte chemoattractant protein

	MD	middle domain
	MHC	major histocompatibility complex
	ML	matrix long protein
	moDC	monocyte-derived dendritic cell
	mRNA	messenger ribonucleic acid
	MVA	modified vaccinia Ankara
	Mx	myxovirus resistance
N	NA	neuraminidase
	NEP	nuclear export protein
	NES	nuclear export signal
	NK	natural killer
	NLRP3	NOD-like receptor family pyrin domain containing 3 protein
	NLS	nuclear localization signal
	NOS2	nitric oxide synthase 2
	NP	nucleoprotein
	NS1	non-structural protein 1
	NS2	non-structural protein 2
	nTreg	natural regulatory T cell
	NXF1	nuclear RNA export factor 1
P	PA	poly acidic
	PB1	poly basic 1
	PB2	poly basic 2
	PBMC	peripheral blood mononuclear cell
	PFU	plaque forming units
	PH	pleckstrin homology
	Poly(A)	poly-adenosine
	PRD	proline-rich domain
	PRR	pattern recognition receptor
Q	QIV	quadrivalent inactivated influenza vaccine
R	RanBP5	Ran-binding protein 5
	RIG-I	retinoic acid-inducible gene I
	RIPK3	receptor-interacting serine/threonine-protein kinase 3

	rMVA	recombinant modified vaccinia Ankara
	RNA	ribonucleic acid
	RSAD2	radical SAM domain-containing 2
	RSV	respiratory syncytial virus
	RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
S	SAM	S-adenosyl methionine
	SARS	severe acute respiratory syndrome
	SEM	standard error of the mean
	SFC	spot-forming cells
	SNP	single nucleotide polymorphism
	SP	surfactant protein
	SPF	specific pathogen free
T	Tcm	central memory T cell
	Tem	effector memory T cell
	Tfh	follicular helper T cell
	Th	T helper cell
	THOV	Thogoto virus
	TIV	trivalent inactivated influenza vaccine
	TMPRSS2	transmembrane protease, serine 2
	TNF	tumor necrosis factor
	TRAIL	TNF-related apoptosis-inducing ligand
	TRIM	tripartite motif
	Treg	regulatory T cell
	Trm	resident memory T cell
V	VHH	single-domain antibody fragment
	VLP	virus-like particle
	vRNA	viral ribonucleic acid
	vRNP	viral ribonucleoprotein
	VSV	vesicular stomatitis virus
W	WHO	world health organization

Empty your mind. Be formless, shapeless, like water. If you put water into a cup, it becomes the cup. You put water into a bottle and it becomes the bottle. You put it in a teapot, it becomes the teapot. Now, water can flow or it can crash. Be water, my friend.

Bruce Lee

PART I: INTRODUCTION

Everything must be made as simple as possible, but not simpler.

Albert Einstein

CHAPTER 1: Influenza A and Thogoto virus

1.1 Classification, nomenclature and clinical impact

Members of the *Orthomyxoviridae* family are characterized by their negative-stranded, segmented ribonucleic acid (RNA) genome. Hitherto, this family contains seven different genera: *Influenza A, B*, *C* and *D*, *Thogotovirus, Quaranjavirus* and *Isavirus*[1-3]. However, novel sequencing techniques and analysis methods suggest the existence of additional viruses that belong to the *Orthomyxoviridae* family, although these viruses have not yet been isolated[4]. The *Thogotovirus* genus can be further divided into seven species: Thogoto, Dhori, Araguari, Upolu, Aransas Bay, Jos and Bourbon virus[1,5-8]. Influenza strains are conventionally named according to genus (A, B, C or D), the animal species – if not human – from which the virus was isolated, followed by the location, number and year of isolation. In the case of influenza A viruses, the hemagglutinin (HA; 1-18) and neuraminidase (NA; 1-11) subtype are also mentioned[9]. For instance, the commonly used lab strain influenza A/Puerto Rico/8/34 (H1N1) is the eighth isolate of a human influenza A virus isolated in Puerto Rico in the year 1934, and it has an HA subtype 1 and an NA subtype 1.

1.1.1 Influenza virus

Every year, seasonal influenza causes an estimated three to five million cases of severe illness, and 250 000 to 500 000 lethal cases worldwide[10]. Next to seasonal influenza, the burden of this disease is also determined by the much feared for and unpredictable pandemic outbreaks of influenza A viruses that carry an HA (sometimes also an NA) that is antigenically very distinct from that of the circulating human influenza viruses. A pandemic can be described as an outbreak which impacts a large geographic area and large proportions of the population in a short period of time. In the past 100 years there were four documented influenza pandemics, namely the Spanish flu (H1N1, 1918), Asian flu (H2N2, 1957), Hong Kong flu (H3N2, 1968) and Mexican flu (H1N1, 2009). The Spanish flu pandemic was by far the most severe of all, killing 25 million people in the first 25 weeks after its outbreak. The most recently declared human influenza pandemic emerged in 2009 and was caused by a swine origin H1N1 virus[11]. It is estimated that globally over 200 000 people succumbed to this virus within the first year after its outbreak in the spring of 2009[12].

1.1.2 Thogoto virus

Thogoto virus (THOV) was first isolated in 1960 from ticks which were themselves isolated from cattle in the Thogoto forest near Nairobi (Kenya)[13,14]. Antibodies against this virus are found in an array of vertebrates such as rats, sheep, cattle, buffaloes, donkeys, camels and humans. In sheep, Thogoto virus has been shown to be a causative agent of febrile illness and abortion[15]. THOV infections in humans are rare, although there is one case where the virus was isolated from the cerebral fluid of a patient with bilateral optic neuritis and in

another case from the blood of a patient with meningitis. However, a causative relationship between the virus and the disease has never been proven[16]. Dhori virus, another tick-borne virus which belongs to the *Thogotovirus* genus, has been shown to cause febrile illness and encephalitis after accidental human lab infections[17]. Bourbon virus, the latest identified member of the *Thogotovirus* genus, recently took its fifth human victim since its discovery[18]. This person was admitted to the hospital suffering from severe headache, pain, a light-red rash, and a low white blood cell count. This was shortly after she removed two ticks from her body.

1.2 Virion and genome structure

Orthomyxoviruses are enveloped viruses whose virions can adopt either a spherical or a filamentous form. Their genome comprises six to eight segments, with some segments coding for more than one protein. Viral RNA sequences of influenza and Thogoto viruses contain conserved sequences at their 3' and 5' ends which are partially complementary[19,20].

1.2.1 Influenza A virus

The outer layer of an influenza A virion consists of a lipid membrane derived from the host cell from which the virion originated. This membrane is studded with viral membrane-spanning proteins *i.e.* HA, NA and a small amount of matrix protein 2 ion channels (M2) (Figure 1.1). HA proteins are essential for the viral attachment to glycans with terminal sialic acids, *e.g.* on host cell glycoproteins, and for the membrane fusion process with the host cell. NA is a sialidase, which is important for cleavage of sialic acids present on virus receptors on the cell surface and on neighboring virions that are budding from an infected cell. NA activity thus ensures efficient release of newly produced virions from the host cell. Furthermore, there is evidence that NA also facilitates the passage of virions through the sialic acid-rich mucosal layer that lines the respiratory mucosa in humans[21]. M2 proteins form tetrameric ion channels in the membrane of the virion and the host cell. The HA:M2 ratio (10^1-10^2 to 1) is much bigger than the HA:NA ratio (4 to 1) due to the small amount of M2 proteins in the virion membrane[22-25]. Just beneath the membrane envelope there is a layer composed of matrix 1 (M1) proteins. This peripheral membrane protein is one of the most abundant proteins in the virion. The M1 protein binds to the lipid envelope and by this maintains the shape of the virion. This shape can be either spherical/elliptical or filamentous[26]. The core of the influenza virion is made up of eight viral ribonucleoproteins (vRNPs), which consist of viral RNA (vRNA) complexed with nucleoprotein (NP) molecules, and a single heterotrimeric RNA polymerase complex comprising the polymerase basic 1 (PB1) and 2 (PB2) and polymerase acidic (PA) proteins. These proteins have their own distinct functions within the viral polymerase, *i.e.* PB1 contains the RNA-dependent RNA polymerase activity[27], PB2 contains the cap-binding activity[28], and the amino-terminal

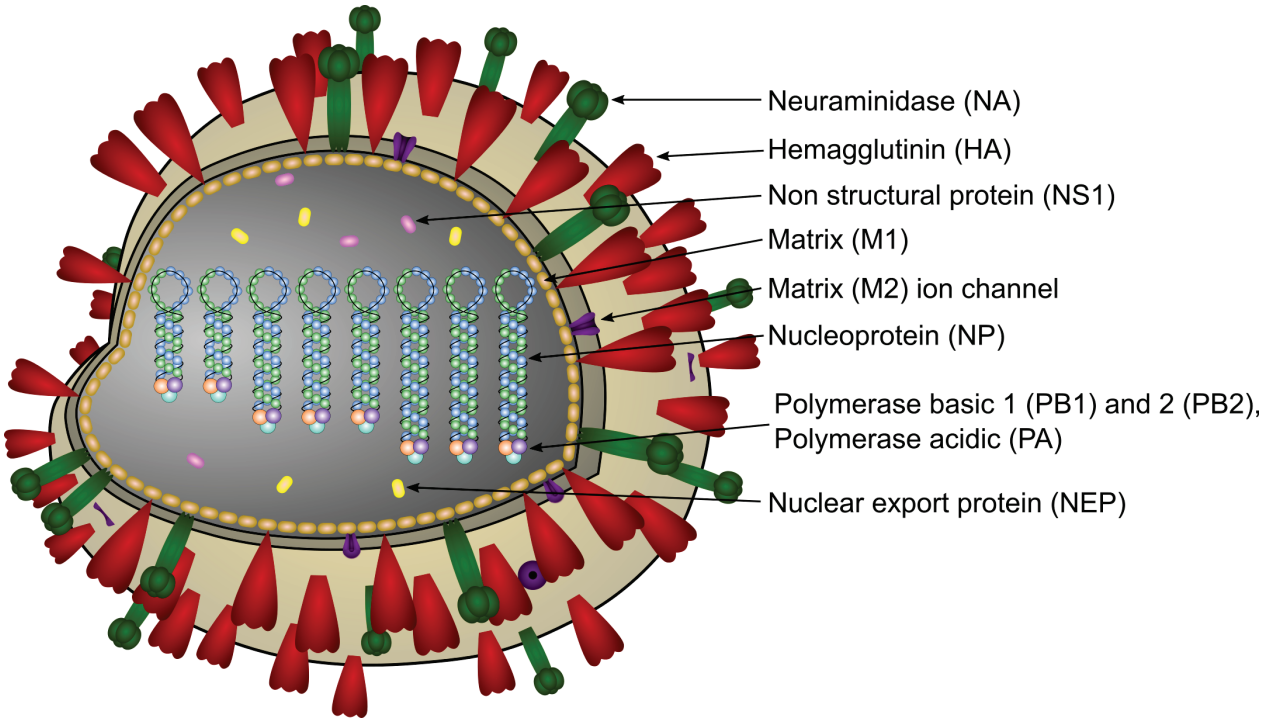

Figure 1.1 Schematic representation of the influenza A virion.

domain of PA functions as an endonuclease[29,30]. Recently, the structures of the influenza A, B and C polymerase complexes were published in a remarkably short period of time[31-33]. Another protein in the virion is the nuclear export protein (NEP), a.k.a. nonstructural protein 2 (NS2), but the exact location of this protein in the virion is not yet known[34-36]. Recently, Hutchinson *et al.* also detected the nonstructural protein 1 (NS1) protein in the virion, albeit at very low levels[25].

The genome of influenza A viruses (FLUAVs) consists of eight segments which code for 13 different proteins. The PB2, PB1, PA, HA, NP, NA, M and NS proteins are encoded by segments 1 to 8 respectively. Spliced and unspliced mRNA transcripts of segments 7 and 8 give rise to different proteins, *i.e.* M1 and M2 for segment 7, and NS1 and NEP (NS2) for segment 8. The NS1 protein interferes with the induction of an adequate interferon response by interacting with different host factors, involved in interferon induction or conveying antiviral action[37-43]. More recently, it has been shown that segment 2 and 3 also code for other proteins next to PB1 and PA respectively. Segment 2 encodes the accessory protein PB1-F2 in a +1 alternate reading frame. This protein has pro-apoptotic activity, regulates host interferon response, and modulates the susceptibility to bacterial infection of the host[44-46]. In addition to PB1 and PB1-F2, segment 2 also codes for a product that is translated starting from an in-frame (relative to PB1) downstream initiation site, producing an N-terminally truncated version of PB1 with no known function, called N40[47]. Segment 3 produces, next to the PA protein, the product of a + 1 ribosomal frameshift *i.e.* PA-X which is

involved in modulation of the host immune response to influenza A virus infection[48]. PB1-F2, N40 and PA-X are not present in the virion of influenza A viruses.

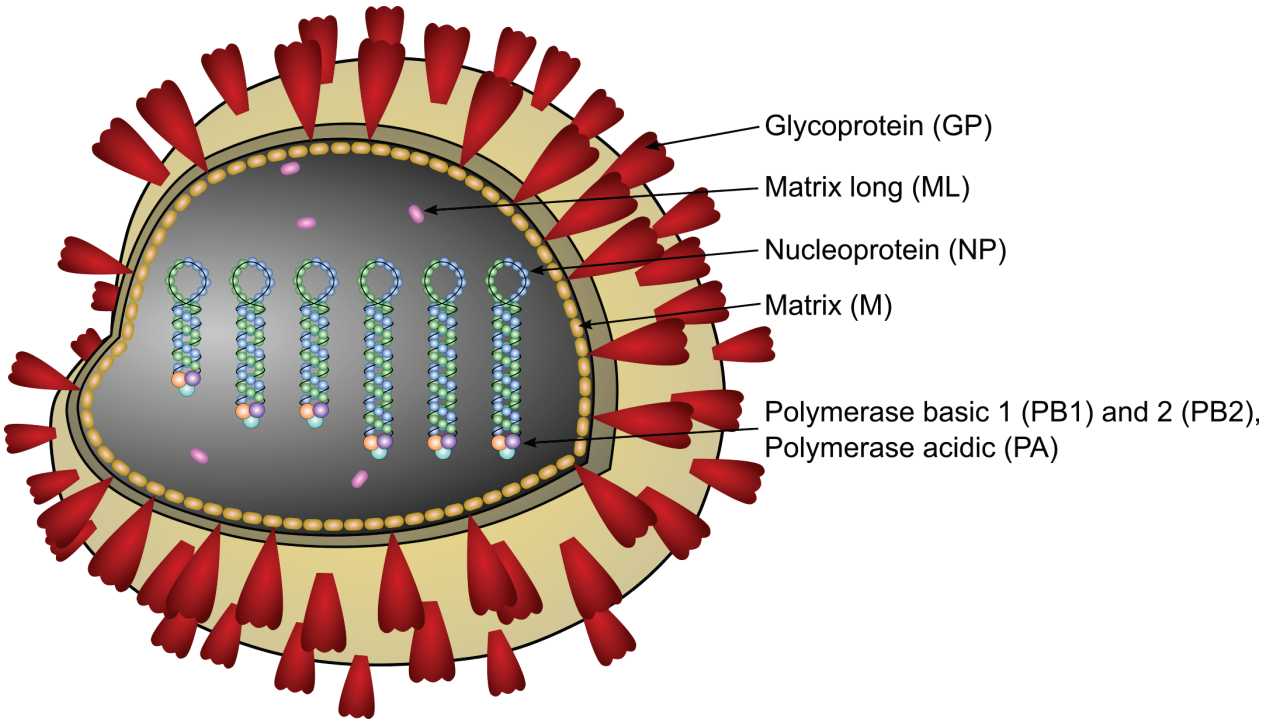

Figure 1.2 Schematic representation of the Thogoto virion.

1.2.2 Thogoto virus

Thogoto viruses resemble influenza A viruses structurally and genetically. The outer layer of the virion is also a host cell-derived lipid membrane studded with glycoproteins (Figure 1.2). The Thogoto virus genome consists of six segments. The three largest segments, similar to influenza A viruses, code for the three RNA polymerase subunits PB2, PB1 and PA. The coding sequences for these polymerase subunits show distant similarity to corresponding sequences of influenza A virus[49,50]. The fourth segment encodes the single glycoprotein (GP) in the THOV envelope. The GP is probably responsible for the virus attachment to and fusion with the host cell. This protein is not related to the influenza A glycoproteins, but there is sequence identity to the baculovirus glycoprotein gp64[51]. Adaptation to the arthropod host is the most plausible explanation for this resemblance. Segment 5 codes for the nucleoprotein (NP), which shows similarity to the corresponding protein of influenza A viruses[52,53]. The smallest segment (segment 6) of the THOV genome codes for two different proteins *i.e.* the matrix (M) and the matrix long (ML) proteins. The M protein is produced from a spliced mRNA[54], like it is also the case for influenza C viruses[55]. This protein has an inhibitory effect on the viral RNA-dependent RNA polymerase and is

essential for the formation of virus-like particles[56]. The ML protein has an extended C-terminus (38 amino acids) in comparison to the M protein, and is translated from the unspliced transcript of segment 6. This protein has been shown to be a potent inhibitor of the interferon β (IFNβ) response, so it fulfills a similar role as the influenza A NS1 protein[57]. Like NS1, the ML protein is also packaged into the virion[56]. The fact that NS1 and ML are structural proteins is in accordance with an interferon antagonizing strategy used by some other viruses: *e.g.* the pp65 protein of human cytomegalovirus and the VP35 protein of Ebola virus counteract the host antiviral response early after infection and are also present in the virion[58,59]. THOV ML suppresses the IFN response in a different way than influenza NS1. FLUAV NS1 prevents IFN induction by retaining the interferon regulatory factor 3 (IRF3) protein into the cytoplasm, by which it cannot reach the nucleus where it would activate the IFNβ promoter[38]. THOV ML, on the other hand, was shown to interact with RNA polymerase II transcription factor IIB. This interaction has a strong negative effect for IRF3-regulated promoters[60].

1.3 Virus replication cycle

1.3.1 Influenza A virus

A schematic overview of the influenza A virus replication cycle is shown in Figure 1.3. Influenza A viruses attach to their host cell by binding to sialic acids on the cell surface. These are commonly found at the termini of many glycoconjugates. Influenza HA glycoproteins on the virion preferentially recognize α2,3-linked and α2,6-linked sialic acids. This means that the carbon-2 of sialic acid is bound either with carbon-3 or -6 of the subsequent galactose molecule, which causes unique steric conformations of this sialic acid. On epithelial cells of the human trachea α2,6-linked sialic acid is the most abundant. However, α2,3-linked sialic acids are also present in the human airways; the highest prevalence of α2,3-linked sialic acids can be found in the lower respiratory tract[61]. On avian gut epithelium cells the most prevalent sialic acids are α2,3-linked. Avian and human influenza strains apparently have evolved to have a preference for either the α2,3- or the α2,6-linked sialic acids respectively[62]. Attachment to the target cell is followed by receptor mediated endocytosis, whereby the virion is engulfed by the target cell and is consequently situated in an endosome.

Following endocytosis, the virus has to release its genetic material into the cytosol. For this, the acidity of the endosome is crucial, since a low pH triggers a conformational change in the HA protein – exposing the fusion peptide – which leads to fusion of the viral and endosomal membrane[63]. The pH of the endosome is also crucial for the acidification of the virion itself. This disrupts the protein-protein interactions between vRNPs and the matrix (M1) proteins, which allows the vRNPs to be released into the host cell cytoplasm. The

acidification of the virion is achieved by the passive transfer of protons into the virus particle through the M2 ion channel. The disruption of vRNP-M1 interactions and the disintegration of the virion matrix layer is a consequence of extensive conformational changes in the M1 structure. A symmetrical dimer conformation is changed into an asymmetrical dimer conformation at low pH[64,65]. The combination of the fusion process and the dissociation of the vRNPs from the M1 matrix proteins allows the release of the vRNPs in the vicinity of the nuclear pores.

Next, the vRNPs traffick into the nucleus by means of their nuclear localization signals (NLSs). All vRNP subunits carry an NLS in their sequence. However, the NP protein was shown to be required and sufficient for import of vRNPs into the nucleus[66,67]. More specific, the unconventional NLS at the amino-terminal end of the NP protein is crucial. Possibly because it is much more accessible than the NLSs of the other subunits of the vRNP complex[68,69]. The NLSs are recognized by importin α proteins which, in turn, are recognized by importin β proteins. The formed complexes are imported into the nucleus by the small GTPase Ran, which shuttles between the nucleus and the cytoplasm[70].

In the nucleus, the viral RNA-dependent RNA polymerase uses the negative sense viral RNA (vRNA) as a template for the production of messenger RNA (mRNA) (viral transcription) and complementary RNA (cRNA) (viral replication). Viral transcription results in the production of positive-stranded mRNA which contains a cap structure at its 5' end, and a poly-adenosine (poly(A)) tail at its 3' end. In this way, the viral mRNA strongly resembles cellular mRNA. The 5' cap is obtained through a process called cap-snatching. The viral polymerase complex 'steals' the cap from cellular pre-mRNA molecules. The cap structure on these pre-mRNAs is recognized and bound by the cap-binding domain of the polymerase PB2 subunit, and subsequently cleaved off – at 10 to 13 bases 3' from the cap structure – by the endonuclease activity of the amino-terminal domain of the PA subunit[28-30]. The cleaved RNA-fragment functions as a primer for initiating the viral transcription by the viral polymerase complex, which synthesizes the viral mRNA as it proceeds over the vRNA template. When the polymerase reaches the 5' end of the vRNA, it forms the poly(A) tail to the 3' end of the viral mRNA by repetitive copying (stuttering) of a stretch of five to seven uracil residues in the vRNA[71-73].

Viral replication is a two-step process wherein firstly the vRNA is copied into a positive-stranded complementary RNA (cRNA), and subsequently this cRNA functions as a template for the production of full-length progeny vRNAs. The initiation of cRNA synthesis occurs *de novo*, and the resulting cRNA does not contain a poly(A) tail. The newly produced cRNA and vRNA are complexed with multiple NP molecules and one polymerase complex, forming

cRNP and vRNP complexes[74]. Therefore, viral replication will only start when a vast amount of free NP molecules are produced. In this way the cRNA is protected from degradation by cellular nucleases[75,76].

Viral mRNAs are transported out of the nucleus similarly to the host mRNA molecules. The two main mRNA nuclear export processes are mediated by the nuclear RNA export factor 1 (NXF1) or by the chromosome region maintenance 1 (CRM1) protein. Which pathway is utilized by influenza viruses is still heavily discussed, but evidence for the NXF1 pathway is growing[77-80]. The NXF1 pathway was shown to be necessary for the optimal nuclear export of influenza mRNA, however the export of PA, PB1 and PB2 mRNA seems to be independent of NXF1 and CRM1[81]. Recently, it was revealed that a member of the DExD-box (DDX) helicases – DDX19 – associates with intronless, unspliced and spliced influenza A mRNAs, and to be involved in their nuclear export[82]. This illustrates the poor understanding about this particular part of the influenza virus life cycle.

After nuclear export, mRNAs are translated to proteins. This is mediated by the eukaryotic translation initiation factor 4 F (eIF4F) complex. This complex consists of three subunits: the cap-binding protein eIF4E, an RNA helicase eIF4A, and a scaffold protein eIF4G[83]. Translation of viral mRNA is reliant on this host cell translation machinery, although some reports suggest that only two out of three subunits are required for this. The eIF4E subunit's cap-binding function is taken over by the viral polymerase PB2 subunit. The eIF4A and eIF4G subunits on the other hand are indispensable for the viral mRNA translation[84,85]. However, based on immune-precipitation experiments, Bier *et al.* proposed that eIF4E (and not the viral RNA polymerase) is involved in cap-binding of influenza A virus mRNA[86].

Envelope proteins are synthesized on endoplasmic reticulum-associated ribosomes and thereby targeted for secretion. In the ER these proteins are folded and N-glycosylated in the case of HA and NA and trafficked to the Golgi apparatus for further post-translational modifications. Eventually, HA, NA and M2 reach the plasma membrane for virion assembly. Newly translated PB1, PB2, PA and NP molecules are imported back into the nucleus where vRNA is encapsidated into vRNPs[87]. The NP protein is transported back into the nucleus by recognition of its unconventional NLS[69]. The polymerase subunits need some extra help to reach the nucleus. The PB2 protein can be assisted by the host cell heat shock protein 90 (Hsp90)[88]. The PB1 and PA polymerase subunits are imported into the nucleus as a heterodimer, helped by the Ran binding protein 5 (RanBP5)[89]. Once arrived in the nucleus, PA, PB1 and PB2 form the polymerase complex. Next to the polymerase subunits and NP proteins, viral NEP and M1 proteins are also transported into the nucleus. The newly

produced vRNPs are exported out of the nucleus and transported to the host cell membrane with the help of M1 and NEP proteins[90]. Since there are no nuclear export signals (NESs) found in RNP proteins, vRNPs need assistance of other viral proteins for nuclear export. M1 proteins bind to the vRNPs and prevent re-entry into the nucleus[91]. M1 subsequently interacts with the NEP protein which does contain an NES. NEP, in turn, interacts with the CRM1 protein that facilitates the nuclear vRNP export[90].

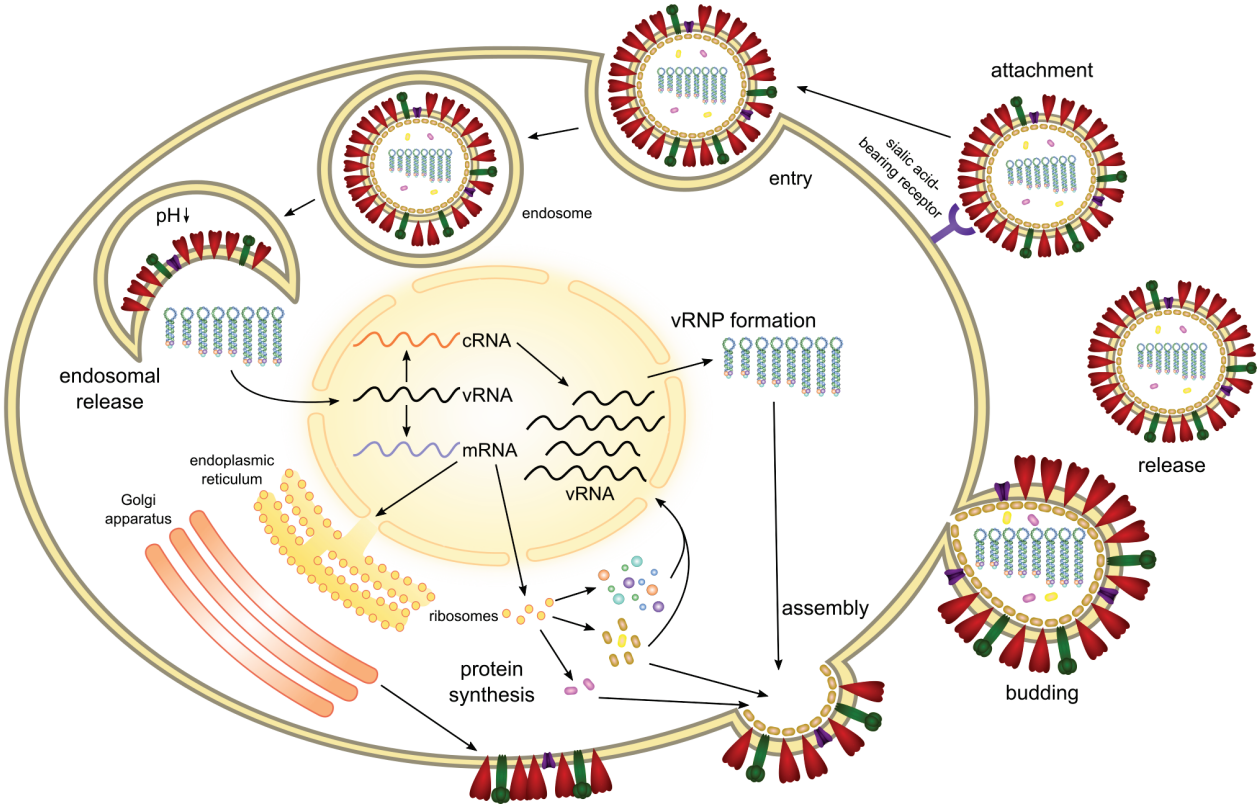

Figure 1.3 Schematic overview of the influenza A virus replication cycle.

Accumulation of HA and NA molecules in lipid rafts (cholesterol and sphingo-lipid enriched areas) in the cell membrane induces virion budding[92]. The cytoplasmic tails of HA and NA proteins function as docking sites for M1 proteins. Here the eight different vRNPs assemble in a unique 7+1 pattern through inter-vRNP and vRNP-M1 interactions[93-95]. Each genomic segment contains specific packaging signals, which facilitate this 7+1 packaging pattern[96]. Subsequent accumulation of M2 in the plasma membrane at the edge of the HA- and NA-containing lipid-rich region, can complete budding by inducing membrane curvature[97,98]. After budding, HA molecules interact with sialic acids on the host cell membrane which would cause them to accumulate on the host cell membrane. To prevent this, NA uses its sialidase activity to cut sialic acids from the host cell glycoconjugates. It also removes the

sialic acids from the virion glycoproteins to prevent aggregation[99]. The newly produced virions are now ready to infect neighboring cells, and start the replication cycle all over.

1.3.2 Thogoto virus

The THOV replication is not as elaborately studied as the influenza A virus replication, but it has been shown that their replication cycles are quite similar. The THOV glycoprotein (GP) functions as a hemagglutinin and as a fusion protein. Fusion is pH dependent, indicating that entry occurs via the endocytic pathway as for FLUAVs[100]. The acidification of the virion also triggers a conformational change in the matrix protein, which supports the process of viral uncoating[101]. After fusion, it has been shown that the THOV replication cycle has a nuclear phase, which suggests that transcription and replication occur in the nucleus[100,102]. Further evidence is provided by the fact that the murine Mx1 protein, which is only active in the cell nucleus, inhibits THOV multiplication[103]. The human MxA protein also inhibits THOV multiplication by preventing the THOV vRNPs from entering the nucleus. MxA inhibits THOV by interaction with the NP molecules of the vRNPs[104,105]. These data suggest that the NP protein plays a major role in the vRNP import. The THOV vRNPs are similar to the ones from FLUAV in the sense that they were shown to be able to transcribe and replicate model vRNA[106]. Like FLUAV, THOV transcription is dependent on the activity of cellular DNA-dependent RNA polymerase II[14,102]. Cap snatching in the FLUAV transcription involves 'stealing' the cap structure of host pre-mRNA with an additional 10 to 13 nucleotides[28-30]. THOV does this slightly differently: the cap structures are still 'stolen' from host pre-mRNA molecules, but there is only one extra nucleotide taken along (preferentially an adenosine residue)[52,107]. Although the THOV and FLUAV polymerase complexes are structurally homologous, their cap-snatching mechanisms probably differ a lot more than previously thought. Guilligay *et al.* showed that the putative PA endonuclease and PB2 cap-binding domains are degenerated and that they have lost their biochemical activities *in vitro*[108]. How cap-snatching is done by THOV remains unclear. The protein synthesis and vRNP formation are not studied extensively for THOV, but are most likely similar to FLUAV considering the similarity between the vRNP-comprising proteins of the two viruses. Budding of the virions is shown to be dependent on GP and M proteins[56,109], as is the case for FLUAV where HA, NA and M proteins are necessary for virion budding.

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And don't spend your time looking around
For something you want that can't be found
When you find out you can live without it
And go along not thinking about it
I'll tell you something true
The bare necessities of life will come to you

Baloo the bear

CHAPTER 2: Myxovirus resistance (Mx) Proteins

2.1 History and phylogeny of Mx proteins

The recorded history of Mx proteins starts in the early 1960s when Jean Lindenmann started investigating the accidental observation of increased resistance against a neurotropic influenza A virus in a line of the mouse A2G strain[1]. Further research showed that these A2G mice are also resistant to other myxoviruses[2]. This feature was passed on to the offspring as a dominant autosomal trait, and the responsible allele was called *Mx*, short for myxovirus resistance[3]. Another landmark experiment was performed by Dr. Otto Haller, who showed that treating A2G mice with an interferon (IFN)-neutralizing antiserum from sheep rendered these mice susceptible to influenza A virus (FLUAV) infection, showing that this resistance associated with the *Mx* allele is interferon-dependent[4]. Building on this, the murine Mx1 protein could be identified as a protein that was only detectable after type I IFN induction of A2G mouse-derived cells[5,6]. Shortly thereafter, a protein – later called MxA – was isolated from human peripheral blood lymphocytes and fibroblasts by immunoprecipitation with a monoclonal antibody (2C12) that had been raised against murine Mx1. This protein was, like murine Mx1, only induced by type I IFN, and not by type II IFN. Unlike Mx1, this protein (MxA) was mainly detected in the cytoplasm[7]. Since then, *Mx* genes have been identified in almost all vertebrates, and all of these *Mx* genes are induced by type I IFN. Notably, an extra *Mx* gene has been identified both in mice and humans, and this gene codes for the Mx2 (mouse) and the MxB (human) proteins respectively[8,9]. More recently, expression of mouse Mx1 and human MxA proteins has been shown to be induced not only by type I, but also by type III IFN[10].

Mx genes are observed in nearly all chordate genomes with few exceptions such as the opossum genome. *Mx* genes can be divided into three phylogenetic classes *i.e.* fish, mammals, and the collective group of amphibians, reptiles and birds[11]. The number of *Mx* genes in fish can vary from one to as many as nine, which is most likely the result of gene duplications. Birds, reptiles and amphibians have only one *Mx* gene, and most mammals have two *Mx* genes (*Mx1* and *Mx2*). From amphibians to mammals the *Mx* alleles are flanked by the same two genes *i.e. TMPRSS2* and *FAM3B*[11]. *TMPRSS2* encodes a serin protease which can process the influenza A HA to a fusion-competent form[12]. *FAM3B* codes for a pancreatic secreted cytokine[13].

The two *Mx* genes in the rodent genome are both closely linked to the *Mx1* genes of other mammals. The *Mx2* gene in rodents is probably derived from the ancestral *Mx1* gene after gene duplication[11]. Most commonly used inbred mouse strains however fail to synthesize a functional Mx1 protein. This is the result of either a deletion of exons 9 to 11, resulting in a frame shift and premature termination of the Mx1 protein (*e.g.* BALB/c and C57BL/6J

strains), or because of a point mutation, which introduces a premature stop codon (*e.g.* CBA/J strain)[14]. Similar gene erosion is seen in the *Mx* genes of toothed whales (*Odontocetes*), which lead to the lack of functional Mx proteins in this phylogenetic group[15]. Next to a defective *Mx1* gene, most mouse inbred strains (including the A2G strain) also carry a defective *Mx2* gene due to a single nucleotide insertion resulting in a frame shift[8]. Markedly, the presence of a functional full-length Mx1 protein does not necessarily convey a strong resistance against FLUAV for a specific mouse strain. The *Mus spretus* Mx1 protein for instance, differing at 25 positions from the *Mus musculus* A2G Mx1 protein, is less potent than A2G Mx1 in inhibiting FLUAV. This difference in potency could be attributed to a single amino acid[16]. The CAST/EiJ mouse strain (derived from *Mus musculus castaneus*) on the other hand, only differs at two positions from the A2G Mx1 protein, but is highly susceptible to FLUAV infection[17,18]. This susceptibility could again be attributed to a single amino acid change[19]. In addition, it has been shown that expression of the A2G *Mx1* gene in a different murine genetic background (DBA/2J) does not result in FLUAV resistance like seen for A2G *Mx1* in the A2G or C57BL/6J background[20]. This can be explained by the highly FLUAV-susceptible phenotype, which is polygenetic, of DBA/2J mice[21]. Furthermore, it was shown that not only the genetic background matters for the protective effect of A2G Mx1 to be displayed, but also the virulence of the virus and the kinetics of Mx1 induction play a major role[20,22,23].

2.2 Structure of Mx proteins

Mx proteins belong to the dynamin superfamily, a subcategory of the large guanosine triphosphate (GTP)-hydrolyzing enzymes (GTPases). All dynamins and dynamin-like proteins consist of at least three domains: a GTPase domain, a middle domain (MD) and a carboxy-terminal GTPase effector domain (GED). Dynamin also contains a pleckstrin homology (PH) domain that facilitates binding to the plasma membrane, and a proline-rich domain (PRD) which can bind other proteins[24]. Mx proteins, however, do not have these PRD and PH domains. The Mx GTPase domain contains a GTP-binding motif and a so-called dynamin signature which is required for GTPase activity[25]. The middle domain of Mx proteins is important for oligomerization and target recognition, and the GED contains an intramolecular GTP-activating domain[26,27] (Figure 2.1).

The three-dimensional (3D) protein structure of MxA was determined in 2011 by Gao *et al.*, and it appeared to strongly resemble the structure of dynamin[28,29]. The 3D structure of MxA can also be divided into three major domains, albeit that these domains differ from the ones recognized in the primary structure. The globular GTPase domain is made up of a central β-sheet surrounded by α-helices. The GTPase domain is connected to the stalk domain, which consists of four α-helices containing the MD and GED[28]. The connection between the GTPase

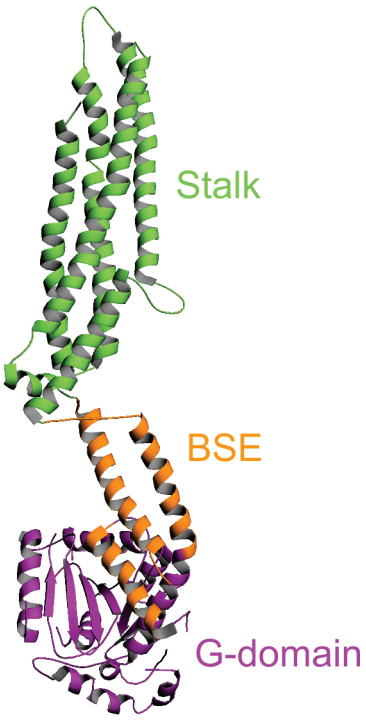

Figure 2.1 Structure of human MxA protein. Ribbon type representation of a human MxA molecule with indication of the three structural domains. BSE = bundle signaling element, G-domain = GTPase domain. Human MxA structure with Protein Data Bank accession code 3SZR. Figure generated using PyMOL software.

domain and the stalk is formed by the third domain called the bundle signaling element (BSE). The BSE comprises three α-helices, each from a different part of the primary structure, and is formed by backfolding of the carboxy-terminal leucin-zipper to the amino-terminal GTPase domain[30].

2.3 Oligomerization of Mx proteins

One of the shared features between dynamin and Mx proteins is their ability to oligomerize and form ring or helical filament structures that surround their target structure (reviewed by Faelber *et al.*[31]). The MxA protein stalk acts as an oligomerization 'hub', with three interfaces and one loop which are responsible for the major interactions between neighboring MxA molecules (Figure 2.2a). First, MxA dimers are formed through a highly conserved interface (interface 2) in the stalk that mediates the dimerization (Figure 2.2b). Further oligomerization results in the formation of a crisscross pattern of MxA molecules by the formation of interactions between the stalk domains of different MxA molecules[26] (Figure 2.3). Additional stabilizing interactions between MxA molecules are mediated by two other interfaces (interfaces 1 and 3) and one loop (L4) located in the stalk region[26,28]. Oligomerized MxA proteins form ring-like structures with the stalks pointed inwards, which is critical for target recognition, and the GTPase domains located at the periphery of the ring[32,33] (Figure 2.4). At the tip of the stalk there is a flexible, unstructured loop (L4) which

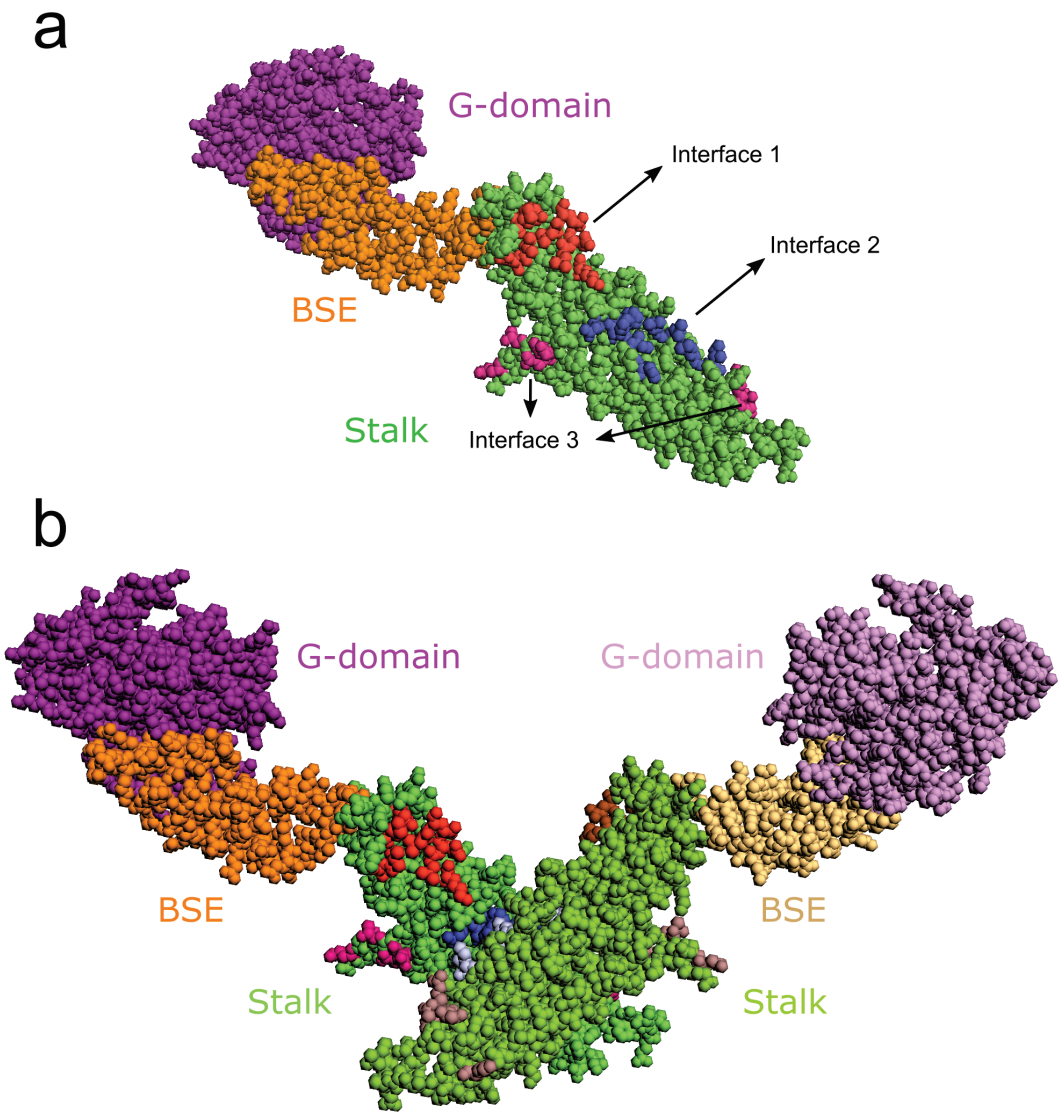

Figure 2.2 Human MxA interaction interfaces and dimerization. (a) Space-filling representation of human MxA with indication of the three interaction interfaces. **(b)** Space-filling representation of a human MxA dimer. BSE = bundle signaling element, G-domain = GTPase domain. Human MxA structure with Protein Data Bank accession code 3SZR. Figure generated using PyMOL software. Figure based on Gao *et al.*, 2010, Nature and Gao *et al.*, 2011, Immunity.

is surface exposed, and which has been shown to be responsible for lipid binding and recognition of certain viral structures[16,34-36]. Ring formation of dynamins has the purpose to facilitate membrane fission. For MxA, this feature is thought to be associated with its antiviral activity[28,37]. A model for dynamin activity was proposed by Faelber *et al.* and Morlot and Roux[38,39], and this model was recently supported with structural, enzymatic and biophysical data gathered from MxA proteins by Rennie *et al.* and Chen *et al.*[40,41]. In this model, MxA's conformation changes drastically upon GTP binding, leading to constriction of the ring or helix, which changes back to the 'resting state' after hydrolysis and release of GDP. This kind of 'power stroke' in a ring formation is essential for dynamin to facilitate

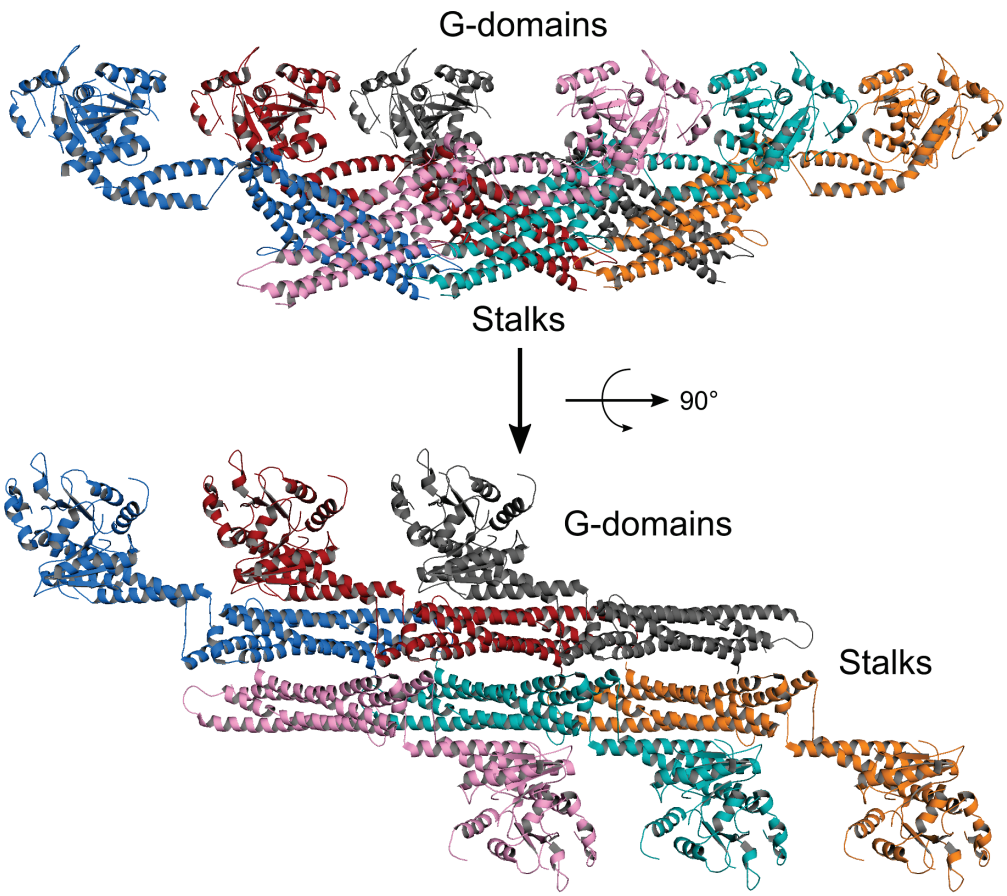

Figure 2.3 Structure of an MxA oligomer. Ribbon type representation of an oligomer formed by six different human MxA monomers. G-domain = GTPase domain. Human MxA structure with Protein Data Bank accession code 3SZR. Figure generated using PyMOL software. Figure based on Gao *et al.*, 2011, Immunity.

scission of membranes (Figure 2.5). However, for MxA-mediated inhibition of Thogoto virus and Vesicular Stomatitis virus (VSV) GTP binding has been shown to be sufficient, so GTPase activity was not necessary[42,43]. This suggests that MxA, at least for some viruses, does not require a mechanical or enzymatic step in its antiviral mechanism.

2.4 Antiviral activity of Mx proteins against Orthomyxoviruses

The antiviral activity of Mx proteins against different members of the Orthomyxoviruses has some common traits. Inhibition of Orthomyxoviruses by Mx proteins requires an intact GTP-binding domain[44]. GTP binding by MxA is thought to facilitate assembly of newly synthesized MxA into stable dimers or tetramers associated with the ER membranes. After interferon induction, and consequently MxA synthesis, the ER membrane acts as a kind of depot for MxA molecules[32,45]. GTP hydrolysis then promotes dynamic redistribution from cellular membranes to viral target structures[46]. Mutations in the GTPase domain can render Mx proteins antivirally inactive against FLUAV[44,47]. However, Janzen *et al.* described an MxA protein carrying a single point mutation (L612K) which caused failure to oligomerize and

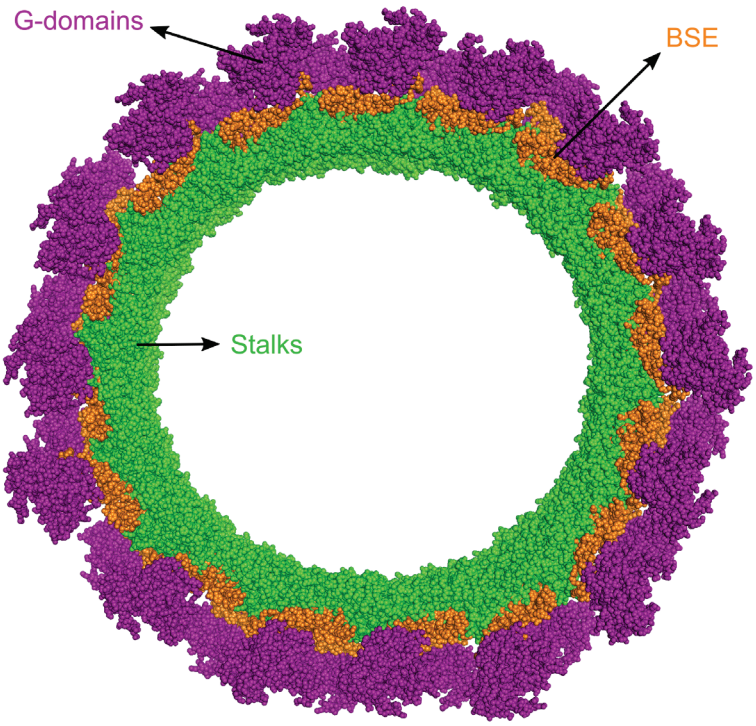

Figure 2.4 Model of ring formed by MxA molecules. Space-filling representation of a ring-like MxA oligomer. BSE = bundle signaling element, G-domain = GTPase domain. Human MxA structure with Protein Data Bank accession code 3SZR. Figure generated using PyMOL software. Figure based on Gao *et al.*, 2011, Immunity.

lack of GTPase activity. Surprisingly, this protein was still able to inhibit THOV and VSV infection[43]. Possibly, this protein could still bind to, but not hydrolyze GTP. Interestingly, GTP binding might not be necessary for recognition of viral targets, but rather stabilizes the complex between MxA and its viral target[46]. However, there is controversy around the notion that monomeric MxA has antiviral activity. The group of Pavlovic reported that overexpressed monomeric MxA can suppress Thogoto virus replication, whereas Chen *et al.* concluded that the monomeric mutant MxA(M527D) does not display antiviral activity[41,48].
Another communal trait of Mx proteins in their inhibition of Orthomyxoviruses is the comparable way of recognizing the virus. This was demonstrated by the fact that the antiviral activity of several Mx proteins (murine Mx1, human MxA, and rat Mx1) can be neutralized with the monoclonal 2C12 antibody which has been raised against murine Mx1[27,49,50]. This antibody targets a conserved epitope in the carboxy-terminal region of Mx proteins[51]. Probably the most important common trait is the importance of the unstructured loop L4, located at the tip of the stalk of Mx molecules. This loop is essential for the specificity and antiviral activity of Mx proteins against Orthomyxoviruses[16,34].

2.4.1 Influenza A virus

Murine Mx1 has a strong inhibitory effect on influenza A viruses, but how this inhibition works exactly remains an enigma. Since mouse Mx1 resides in the nucleus[52], it presumably has no effect on viral attachment and entrance, uncoating of the virus, or transport of vRNPs to the nucleus[6,53,54]. Instead, the mouse Mx1 activity specifically targets primary viral transcription. Murine Mx1 has been shown to differentially inhibit the transcription of diverse influenza genomic segments[55]. Mainly the larger segments, such as PB2, PB1 and PA, are affected. The effect on the HA and NP segments is less pronounced, and is almost nonexistent for the smallest segments *i.e.* M and NS. This indicates that murine Mx1 primarily interferes with transcriptional elongation instead of initiation[55]. The main targets of Mx1 are the NP and the PB2 proteins. Both are components of the influenza vRNPs, and together with PB1 and PA, essential for influenza transcription. Overexpression of the PB2 protein can outcompete Mx1 activity[56,57], and NP has been shown to determine the sensitivity of influenza A viruses for Mx1 and MxA activity[37,47,58]. The latter is the reason why human influenza A viruses are more resistant to murine Mx1 or human MxA activity than avian influenza A viruses. This reduced sensitivity can be attributed to only three exposed residues in the body domain of NP which are accessible for Mx proteins[59,60]. Introducing this 'Mx escape cluster' into the NP of otherwise Mx-susceptible viruses drastically reduces viral fitness of these viruses[59], suggesting that escape from human Mx activity and maintaining viral fitness proves to be challenging for influenza viruses. Because of this, Mx proteins are considered a powerful barrier against zoonotic transmission of avian influenza viruses. The newly emerging H7N9 FLUAV however overcomes this restriction with an NP that is less sensitive to the activity of Mx[61]. Interestingly, Hanke *et al.* recently described an NP-specific single-domain antibody fragment (VHH) that binds a region on the body domain of NP, and this region overlaps with the cluster of residues which are decisive for Mx1 and MxA sensitivity. Based on a reporter assay, wherein the VHH and murine Mx1 differentially affected the expression of long and short transcripts, the authors concluded that this VHH displayed similar inhibitory effects on the influenza virus transcriptional elongation as seen for murine Mx1[62]. This indicates that mere binding to NP molecules can be sufficient to interfere with their role in transcription elongation. Our lab demonstrated that Mx1 can interact with PB2 and NP, and moreover, that Mx1 inhibits the interaction between PB2 and NP molecules[37]. Not only can Mx1 inhibit the PB2-NP interaction, it might actively disrupt this interaction in already formed vRNPs. Our lab concluded this based on experiments with a synthetic murine Mx1 construct which could be conditionally activated. Activation of the Mx1 construct after formation of FLUAV vRNPs still resulted in disruption of the PB2-NP interaction, and consequently resulted in the inhibition of FLUAV replication[63].

Unlike mouse Mx1, human MxA is located in the cytoplasm, thus it can inhibit both viruses that replicate in the cytoplasm and those that replicate in the nucleus (antiviral profile of MxA reviewed by Verhelst *et al.* and Haller *et al.*[11,64]). Inhibition of influenza A virus infection by MxA was initially thought to occur after primary viral transcription, but before viral proteins are expressed[55,65]. However, Xiao *et al.* showed in *in vitro* studies that MxA – in cooperation with interferon-induced transmembrane 3 (IFITM3) protein – retains viral genomic RNA in the cytoplasm near the late endosome[66]. If only MxA is present, which was accomplished by the use of a stably transformed cell line that constitutively expresses MxA, influenza A vRNPs can still migrate to the nucleus and start viral transcription, but the viral

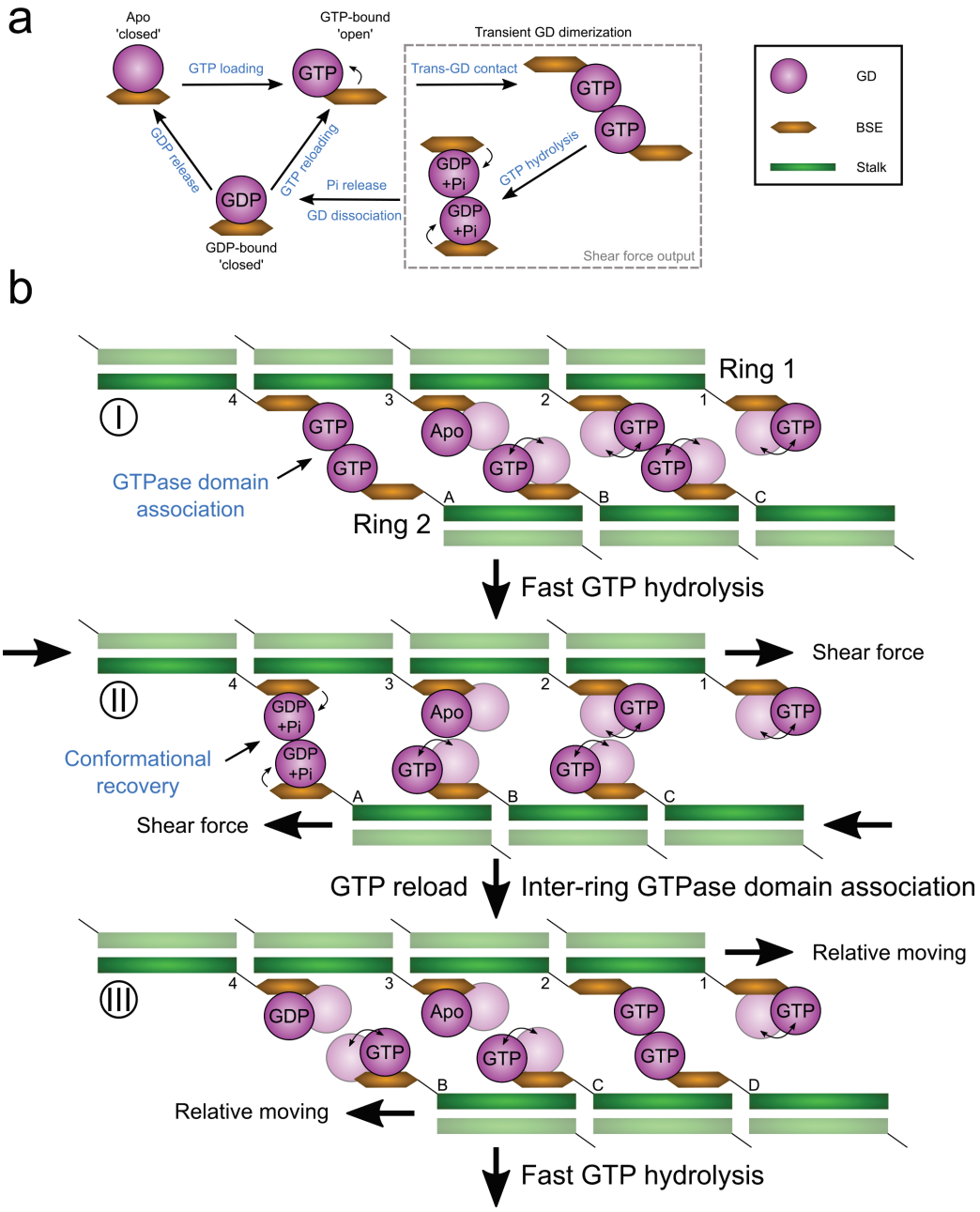

Figure 2.5 Mechanism of polymer constriction by dynamin or MxA rings. (a) Schematic representation of the process of shear force output in the GTP hydrolysis cycle. **(b)** GTPase domains interact randomly, and subsequent GTP hydrolysis induces power strokes which drives conformational changes in the MxA/dynamin molecule. These conformational changes cause the movement of the MxA/dynamin polymers in the opposite direction, hereby constricting the vRNP or the membranes of the budding vesicle respectively. Two neighboring rings are represented as two linear oligomers. Opposing molecules from upper and lower oligomers are numbered 1-4 and A-D, respectively. (I) GTP-binding shifts the GD from a closed to an open conformation. In this conformation, GDs from molecules 4 and A associate with each other. (II) GTP hydrolysis induces the GDs of molecules 4 and A to return to the closed conformation. This so-called power stroke generates a latitudinal shear force. (III) This shear force promotes the relative moving of the neighboring rings in opposite directions. GDs are reloaded with GTP, and again may randomly form contact with GDs from the neighboring ring. The whole process is constantly repeated, generating a continuous shear force and consequently movement of the neighboring rings. GD = GTPase domain, BSE = bundle signaling element. Figure adapted from Chen *et al.*, 2017, Nature Communications.

genome is not replicated[66]. So it is tempting to hypothesize that human MxA only prevents nuclear import of newly synthesized vRNP components, but until today this has not been confirmed. Interestingly, fusion of MxA with an NLS showed that the working mechanism of MxA is similar to that of murine Mx1 in the nucleus[52,67]. Here, it was also postulated that MxA interacts with PB2 and NP, like murine Mx1. This was concluded based on two findings, first, overexpression of PB2 or NP could counteract the inhibition by nuclear MxA. Second, NP co-immunoprecipitates with nuclear MxA and wild type MxA, albeit after the used cell lysates were treated with a reversible cross-linking reagent prior to immunoprecipitation[67]. Considering this, another hypothesis can come to mind wherein human MxA can inhibit influenza A virus by recognizing the incoming vRNPs and form oligomeric rings around them at the cytoplasmic side of the nuclear membrane, and mouse Mx1 could do the same at the nuclear side of the membrane. This would mean that Mx proteins in influenza A virus infected cells act as some kind of gatekeepers to the nucleus[11].

2.4.2 Thogoto virus

Thogoto virus is very sensitive to Mx1 and MxA activity[68,69]. The exact mechanism of THOV inhibition by mouse Mx1 has not been studied in detail. However, fusion of an NLS to human MxA blocks the THOV polymerase activity in the nucleus by interacting with the assembled vRNPs[70]. Given the fact that mouse Mx1 and the nuclear form of MxA show a similar working mechanism for the inhibition of FLUAV, and that FLUAV and THOV show a lot of similarities in their replication cycles, it is conceivable that mouse Mx1 inhibits the primary transcription of THOV in the same way it does for FLUAV.

MxA can block the transport of THOV vRNPs to the nucleus by interacting with the NP components of the vRNPs, and so covering the NLSs of the vRNPs[71]. The region of MxA that interacts with NP is located at the carboxy-terminal domain which contains loop L4[34,71]. This was demonstrated through preventing the interaction between MxA and NP by adding

the monoclonal 2C12 antibody who's epitope is located at the carboxy-terminal end of MxA[68]. Loop L4 is necessary for the interaction between MxA and NP, and by extension for the antiviral activity against THOV and FLUAV. Especially the phenylalanine residue at position 561 in this loop is essential for the antiviral activity of human MxA against THOV and FLUAV[34].

2.5 MxB: The new kid on the block

Until recently it was thought that the human MxB protein had no antiviral role, and that it was solely involved in cell cycle progression[72]. However, this was refuted concomitantly by two research groups. Their work was based on the finding that type I IFN treatment can reduce human immunodeficiency virus type 1 (HIV-1) infection in cultured human cells and cell lines[73,74]. Transcriptional profiling of RNA isolated from these treated cells revealed several candidate interferon-stimulated genes (ISGs) that suppressed HIV-1 infection. After testing these candidate genes *in vitro*, only the human MxB exhibited a clear antiviral phenotype against HIV-1 infection[75,76]. These results were backed up by studies where the antiviral properties of several ISGs were assessed, proving that MxB displayed antiviral activity against vesicular stomatitis virus, mouse herpes virus type 68, and HIV-1[77-79]. Further research demonstrated that human MxB blocks nuclear uptake of HIV-1 viral replication complexes[75,76]. It most likely does this by inhibiting the uncoating process of HIV-1, and hereby 'trapping' the proviral DNA in the virus core after reverse transcription occurred[80]. Thereby, chromosomal integration – a key event of the HIV-1 replication cycle – is inhibited. Inhibition of the uncoating process is mediated by binding of MxB to the capsid protein of HIV-1, which was readily indentified as the specific target and restriction factor for the antiviral activity of MxB[75,76,79,81]. The amino-terminal region of MxB was shown to be responsible for binding the capsid protein[76,82-86]. More precisely, a triple-arginine motif within the amino-terminal region (at positions 11 to 13) is essential for HIV-1 capsid-binding, and by extension for anti-HIV-1 function[83,86]. This explains why the shorter isoform of MxB, which lacks the first 25 residues of the amino-terminal domain (including the triple-arginine motif), does not affect HIV-1 infectivity[76,84]. In addition to the amino-terminal domain, there is one other factor which is very important to ensure antiviral activity of the MxB protein. Oligomerization[87], and more specific dimerization, seemed indispensable for robust inhibition of HIV-1 infection[82,83,88,89]. However, Alvarez *et al.* recently studied MxB oligomerization in detail by using cryo-electron microscopy and a fusion protein of maltose binding protein and MxB. Their data suggest that nonhelical MxB oligomers greater than a dimer are the active anti-HIV-1 species, and that a newly discovered oligomerization interface is crucial for MxB oligomerization, and consequently for antiviral function[90].

Clinical data were gathered that suggest an important role for the MxB protein in the resistance against HIV-1 infections. The human *MX2* gene is represented by two different haplotypes in Asian and European populations. The G allele of haplotype rs2074560(G/A) is enriched in a cohort of individuals who were protected from HIV-1 infection after repeated exposure to the virus[91]. On the other hand, naturally occurring capsid variants can render HIV-1 resistant to the effects of MxB. Knowing this, it is quite alarming that these variants are most frequently found in clade C of HIV-1, which is the most rapidly expanding clade throughout the world[92].

Recently, also herpes viruses were shown to be sensitive to MxB activity. This viral restriction shows similarities with the anti-HIV-1 activity of MxB in that only the long isoform of MxB is able to inhibit herpes viruses, indicating that the amino-terminal domain is a key determinant for antiviral activity. Oligomerization is, like in HIV-1 restriction, also important for the inhibition of herpes virus infection. In contrast to its anti-HIV-1 activity, MxB seemed to require a functional GTPase domain for its anti-herpes virus activity[93].

2.6 Known role for Mx1 protein in the immune cell compartment

The role of the Mx1 protein in the immune cell compartment is poorly characterized. For a start, almost all immunological studies in life science research labs are performed in inbred mouse strains that lack a functional *Mx1* gene. However, there were some studies on the possible role of Mx1 in immune cells for viral resistance. Haller and coworkers showed that athymic (nude) mice, carrying a functional *Mx1* gene, survived intracerebral infection with a neurotropic influenza A virus strain, leading them to conclude that mice that carry a functional *Mx1* gene do not require a functional T cell system or an orderly development of neutralizing antibodies to survive a primary infection with influenza A virus[94]. Suppression of the immune system with either cyclophosphamide, methotrexate, or procarbazine showed no effect on the resistance of *Mx1*-bearing mice against the highly pathogenic hepatotropic influenza A virus strain TURH (derived from the avian influenza A virus strain A/Turkey/England/63)[95]. Some years later, the same lab demonstrated that *in vivo* resistance to a macrophage-adapted influenza A virus was largely independent of whether or not the mice's macrophages carried a functional *Mx1* gene[96]. These studies led to the assumption that *Mx1*-expression in immune cells does not play a major role in the resistance against influenza virus infection. However, several studies have reported different, sometimes contradictory, findings about the ability of influenza viruses to infect different immune cell types and the formation of a type I IFN-dependent antiviral state in these cells. Moltedo and colleagues state that migratory $CD103^+$ dendritic cells (DCs) can be infected and carry infectious virus from lungs to the mediastinal lymph nodes. This is possible because of an attenuated interferon α/β receptor (IFNAR)-response[97]. Another

study, however, claims that CD103$^+$ DCs are not productively infected. This study shows that CD103$^+$ DCs are resistant to influenza virus infections by acquiring an antiviral state, which is induced by type I IFN signalization[98]. In this respect, Wakim *et al.* showed that lung resident memory CD8$^+$ T cells express elevated levels of the interferon-induced transmembrane protein 3 (IFITM3), which also displays antiviral activity. This elevates the resistance of these cells against influenza virus infection[99]. So it seems that interferon signaling and interferon-induced proteins play a major role in determining whether certain immune cell types are susceptible or resistant to influenza virus infection. However, lung macrophages block seasonal influenza virus and do not allow productive replication, but this block is supposedly not dependent on interferon-induced proteins such as IFITM3 or Mx1 because they affect other steps in the replication cycle than the ones where the blockages in macrophages were observed[100]. Nevertheless, all these studies have been performed in mice or cells that do not express a functional form of the Mx1 protein, which is a powerful antiviral mediator. So one could argue that completely ignoring a role for Mx1 in immune cells, based on these studies, would be putting things too bluntly.

2.7 Interferon-stimulated genes and their anti-influenza A virus activity

Next to Mx proteins, the variety of interferon-stimulated gene products with antiviral activities is ample. Here, a brief overview is given of the interferon-stimulated genes (ISGs) with known anti-influenza activity.

2.7.1 OAS/RNaseL system

The oligoadenylate synthetase (OAS)/RNaseL system was discovered in the late 1970s[101]. The activity of RNaseL is dependent on IFN, and it is correlated with the synthesis of 2'-5' oligoadenylates (2-5A) by OAS. This synthesis is triggered by binding of double-stranded RNA (dsRNA) to OAS, which then converts ATP to 2-5A. These 2-5A are short oligoadenylates linked by 2', 5'-phosphodiester bonds, and their only function known today is the activation of RNaseL[102]. RNaseL is a latent endoribonuclease which cleaves viral and cellular mRNA. RNaseL is activated by forming homodimers, this dimerization is triggered by 2-5A. Activated RNaseL cleaves viral and cellular single-stranded RNA (ssRNA), which is a threat for RNA viruses such as FLUAV[103]. However, FLUAV is not strongly affected by the RNaseL activity. This is because FLUAV produces the NS1 protein which binds to dsRNA, and isolates it from OAS. Therefore, OAS activation and subsequent RNaseL activation is prevented[104].

2.7.2 Double-stranded RNA-activated protein kinase

Double-stranded RNA-activated protein kinase (PKR) (or eukaryotic translation initiation factor 2-alpha kinase (IEF2AK2)) is a known inhibitor of cellular and viral mRNA translation. It is constitutively expressed at low levels in mammalian cells. In resting

conditions it resides in the cytoplasm as an inactive monomer[105]. Upon binding of dsRNA it forms homodimers and performs autophosphorylation which stabilizes the now active dimeric enzyme[106,107]. Activated PKR catalyzes the phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α) which, in turn, inhibits mRNA translation. In this way it hampers the cellular and viral protein synthesis. As with RNaseL, FLUAV has a way to circumvent the inhibitory effects of PKR by means of its NS1 protein. The sequestration of dsRNA by NS1 also prevents PKR from getting activated. Additionally, NS1 can form a complex with PKR and in this way directly inhibit the enzyme[108].

2.7.3 Cholesterol-25-hydroxylase

Cholesterol-25-hydroxylase (CH25H) is an enzyme that converts cholesterol into 25-hydroxycholesterol (25HC), and whose expression is upregulated in response to type I and II IFN[109-111]. 25HC, the product of CH25H-activity, protects cells against viral infection by inducing changes in the physical properties of host cell membranes. High 25HC concentrations prevent fusion of virus and host membranes, and consequently prevent virus entry[112]. Therefore, it is no surprise that enveloped viruses – including influenza A virus – are sensitive to the antiviral activity of 25HC[112,113].

2.7.4 TRIM proteins

The tripartite motif (TRIM) protein family contains more than 60 members with a wide range of activities. The best known TRIM protein probably is TRIM5α, which has an anti-HIV-1 activity. TRIM5α accelerates the uncoating process of the HIV-1 virion by direct binding to the viral capsid proteins, hereby prematurely exposing the HIV-1 nucleoprotein complex[114]. Next to TRIM5α, the TRIM protein family has other members with antiviral activities. Of interest is the TRIM22 protein, which also plays a role in the restriction of HIV-1 virus infection[115] and, in addition, inhibits hepatitis B virus[116], encephalomyocarditis virus[117], and influenza A virus infection[118]. TRIM22 restricts influenza A virus infection by targeting the virus nucleoprotein for proteasomal degradation.

2.7.5 IFITM proteins

Interferon-induced transmembrane (IFITM) proteins were first described based on their expression after IFN treatment of cells[119]. Humans express four different IFITM proteins, IFITM1, -2, -3 and -5, whereas mice express two additional IFITM proteins, IFITM6 and -7[120]. IFITM1 to -3 are nearly ubiquitously expressed throughout all cell types. IFITM5 however, is solely expressed in osteoblasts where it has a role in bone mineralization[121,122]. Members of the IFITM protein family inhibit FLUAV, HIV-1, hepatitis C virus (HCV), respiratory syncytial virus (RSV), severe acute respiratory syndrome (SARS) coronavirus, and members of the Flavi-, Filo-, Rhabdo-, Bunya-, and Reoviruses (reviewed by Bailey *et*

al.[120]). Regarding influenza A virus infection, IFITM3 shows the most potent antiviral effect[123].

2.7.5.1 IFITM3

Next to Mx1/MxA, IFITM3 is also a major contributor to the resistance against influenza A virus infections[123,124]. Xiao and colleagues observed an additive antiviral effect when both proteins were coexpressed in FLUAV-infected cells[66]. Moreover, it was postulated that MxA cannot fully compensate for the loss of IFITM3 in IFN-treated cells challenged with influenza A virus[125]. A possible explanation might be the evolution of human influenza strains to evade the effects of MxA[47,58], rendering the cell reliant on an additional antiviral protein/mechanism, in this case IFITM3. The importance of IFITM3 became clear when Everitt *et al.* assessed the *IFITM3* alleles of patients hospitalized with seasonal or pandemic H1N1/09 influenza A virus infections[123]. They found an enrichment of a minor *IFITM3* allele (SNP rs12252-C) with an altered splice acceptor site. This alteration may be associated with a splice variant of IFITM3 lacking the first 21 amino-terminal residues due to use of an alternative start codon. This minority variant of IFITM3 was less well expressed, and made cells more susceptible to influenza virus infection *in vitro*. However, Kim *et al.* recently observed a higher prevalence of the C-allele of the rs12252 SNP in a Korean cohort, but this was not coupled to a higher prevalence of severe disease and death in this cohort. This suggests that this SNP in the *IFITM3* gene may not be a determinant of disease severity of FLUAV infection after all[126]. Nevertheless, another SNP (rs34481144) was recently discovered in the 5' untranslated region of the *IFITM3* gene. The A allele is correlated with severe influenza infections as shown in three independent cohorts. This so-called risk allele disrupts a CpG site which undergoes differential methylation in $CD8^+$ T cell subsets which leads to reduced levels of $CD8^+$ T cells in the airways after FLUAV infection[127].

IFITM3 resides in the membranes of late endosomes and lysosomes, where it prevents fusion between virus and host membranes. By this, it traps viruses in an environment which will ultimately destroy them due to its degradative properties[125]. Thus, IFITM3 allows viruses to be taken up in endosomes, but inhibits the viral fusion and subsequent release of viral contents into the cytosol[125,128,129]. Since IFITM3 only resides in late endosomes, viruses which fuse with early endosomal membranes might be insensitive to IFITM3 activity[125]. Furthermore, recent work by Gerlach and coworkers revealed that influenza A viruses with a higher pH optimum for fusion are more resistant to IFITM3[130]. This is not unexpected, seeing that the pH in early endosomes is higher than the pH in late endosomes. In contrast, Weston *et al.* recently observed that IFITM3 proteins mainly reside in early endosomes in A549 cells, and that they can inhibit Semliki Forest and Sindbis virus

infection[131]. These are alphaviruses known to enter the cell cytoplasm by membrane fusion with early endosomes rather than late endosomes.

The role of IFITM3 as an innate component of the antiviral immune response was expanded when Wakim *et al.* suggested a role for IFITM3 in the immune cell compartment[99]. After challenging mice with a sublethal dose of influenza A virus, lung-resident memory $CD8^+$ T cells retained a high expression of the IFITM3 protein. Due to hypomethylation of the *IFITM3* promoter in these cells, they selectively retain IFITM3 expression and are therefore better protected against infection with FLUAV and other viruses. Recently, a comparable observation was reported in human subjects where memory $CD8^+$ T cells also retain IFITM3 expression after FLUAV infection. However, this was caused by a hypermethylation of the *IFITM3* promoter instead of a hypomethylation[127]. Thus, IFITM3 fulfills an important prophylactic function in the immune cell compartment by providing (lung resident) memory $CD8^+$ T cells with greater resistance to viruses. One could be tempted to argue that other ISGs might have similar functions within the immune cell compartment.

2.7.6 ISG15

ISG15 is a ubiquitin-like protein which can be covalently attached to several target proteins in a process called ISGylation[132]. ISGylation has different effects depending on the targeted protein, *e.g.* the stability of an ISGylated protein can either be increased or decreased[133,134], or its affinity to its target can be enhanced[135]. It has been shown that ISG15 is preferentially conjugated to newly synthesized proteins, and this might be a general, nonspecific mechanism of host defense. By this, ISG15 potentially has an impact on all viral proteins translated in IFN-stimulated cells[136]. This is also the case for FLUAV proteins, as observed for the NS1 protein[137,138]. According to these observations, it is not unexpected that *ISG15*$^{-/-}$ mice are more susceptible to FLUAV infections[139,140].

2.7.7 Viperin

Viperin (or radical S-adenosyl methionine (SAM) domain-containing 2 (RSAD2)) is a virus inhibitory protein which normally resides in the endoplasmic reticulum (ER) and ER-derived lipid droplets. Viperin inhibits an array of enveloped viruses by inhibiting the enzyme farnesyl diphosphate synthase (FPPS), an enzyme involved in the isoprenoid synthesis. The decrease in FPPS activity alters the fluidity of host cell membranes, and as such hampers the budding of FLUAV by perturbing the lipid rafts where FLUAV budding occurs[141]. However, in contrast with these observations, *Rsad2*$^{-/-}$ and wild type mice challenged with FLUAV reacted similarly regarding mortality, lung pathology, and viral titers[142].

2.7.8 Tetherin

Tetherin (or bone marrow stromal antigen 2 (BST2)) also shows antiviral activity against a number of enveloped viruses. More specifically, tetherin inhibits virus budding. It was shown to inhibit budding of FLUAV virus-like particles (VLPs)[143,144], but the ability to inhibit budding of infectious virus is rather doubtful[143,145,146]. In contradiction with these observations, the influenza NA protein antagonizes tetherin partially through an unknown mechanism[146], which would suggest that during the evolution of FLUAV a mechanism was created to oppose the activity of tetherin.

2.7.9 ZBP1

Z-DNA binding protein 1 (ZBP1 or DNA-dependent activator of IFN regulatory factors (DAI)) is a common upstream regulator of the NOD-like receptor family pyrin domain containing 3 protein (NLRP3) inflammasome and various programmed cell death pathways during influenza virus infection[147-149]. Influenza infection triggers a type I IFN response which induces the production of ZBP1 in an IFNAR1-, STAT1- and IRF9-dependent manner[147]. ZBP1 then interacts with receptor-interacting serine/threonine-protein kinase 3 (RIPK3), and this ZBP1-RIPK3 complex is responsible for the activation of the NLRP3 inflammasome. This inflammasome is a multiprotein complex consisting of NLRP3, adaptor protein apoptosis-associated speck-like protein containing a CARD domain (ASC) and a cysteine protease called caspase-1. Inflammasome assembly activates caspase-1 which, in turn, will mediate processing of the proinflammatory cytokines IL-1β and IL-18. Active caspase-1 can also induce an inflammatory form of cell death called pyroptosis which is executed via its substrate gasdermin D (reviewed by Kuriakose *et al.*[150]). How ZBP1 is activated is still a heavily debated topic. Kuriakose and colleagues showed that ZBP1 can interact with the FLUAV proteins PB1 and NP, and they concluded that ZBP1 is a sensor of IAV proteins that can trigger both cell death and inflammatory responses during influenza virus infection[147]. In contrast, Thapa *et al.* reported that ZBP1 senses FLUAV RNA instead of FLUAV proteins[148]. The latter was consolidated by Maelfait *et al.* whose data, which were gathered with a different infection model, strongly suggest that ZBP1 is an RNA sensor[151]. However, Kesavardhana and coworkers recently reported that both FLUAV proteins PB1 and NP, and the FLUAV RNA genome are interacting partners of ZBP1 during FLUAV infection[152]. Conceivably, ZBP1 mainly senses viral RNA in the vRNPs, and binds indirectly with FLUAV PB1 and NP which are also components of this vRNP.

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Science knows it doesn't know everything; otherwise it'd stop.

Dara Ó Briain

CHAPTER 3: Influenza and memory T cells

Influenza and Memory T Cells: How to Awake the Force

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Abstract

Annual influenza vaccination is an effective way to prevent human influenza. Current vaccines are mainly focused on eliciting a strain-matched humoral immune response, requiring yearly updates, and do not provide protection for all vaccinated individuals. The past few years, the importance of cellular immunity, and especially memory T cells, in long-lived protection against influenza virus has become clear. To overcome the shortcomings of current influenza vaccines, eliciting both humoral and cellular immunity is imperative. Today, several new vaccines such as infection-permissive and recombinant T cell inducing vaccines, are being developed and show promising results. These vaccines will allow us to stay several steps ahead of the constantly evolving influenza virus.

3.1 Introduction

Influenza virus infection induces a profound humoral and cellular immune response in the host. Serum IgG antibodies that are directed to influenza virus hemagglutinin (HA) and neuraminidase (NA) glycoproteins correlate with protective immunity[1,2]. Influenza viruses evade this humoral response by a process named "antigenic drift". This phenomenon is driven by the relatively high frequency of misincorporated nucleotides into the genomes of the viral progeny by the viral RNA-dependent RNA polymerase complex[3]. Immune pressure exerted by antibodies that inhibit HA or NA function can lead to the selection of viruses from this diverse genetic pool that can escape humoral immunity. Cellular immunity on the other hand is mainly directed against more conserved internal influenza viral proteins such as the nucleoprotein (NP)[4]. Within this arm of the adaptive immune system, $CD8^+$ T cells are very important for virus clearance, and are able to provide heterosubtypic immunity[5]. The importance of $CD8^+$ T cell immunity in the host control of influenza virus infection is illustrated by the higher incidence of mutations in the T cell epitope regions of e.g., NP compared to the rest of the protein, which indicates that these epitopes are under selective pressure[6,7].

Annual vaccination is considered the most effective way to prevent disease caused by influenza A and B virus infection[8]. In many countries this practice is recommended for all persons older than six months who do not have contraindications, and especially for elderly people, immunocompromised persons and children[9]. The virus strains that are included in these vaccines are recommended by an expert panel of the World Health Organization (WHO) that bases its predictions on influenza surveillance data (Global Influenza Surveillance and Response System).

Most of the current licensed influenza vaccines are tri-(TIV) or quadrivalent (QIV) inactivated formulations that contain 15 μg each of the hemagglutinin glycoprotein of two influenza A strains (H1N1 and H3N2) and one or two influenza B strains (Yamagata and Victoria lineage). Inactivated influenza vaccines are administered by intramuscular injection or, less frequently, by intradermal injection. Mostly, these types of vaccines are derived from viruses grown in fertilized chicken eggs. However, it is worth mentioning Flucelvax®, which is an FDA approved TIV that is manufactured using mammalian cell culture technology[10]. Alternatively, a live-attenuated influenza vaccine (LAIV) that is administered intranasally is available in the USA and some European countries. This vaccine is licensed for use in healthy individuals aged 2–49 years[9]. This vaccine is produced by reassortment of the selected influenza virus strain with the cold-adapted A/Ann Arbor/6/1960 vaccine strain. It only replicates efficiently at a temperature of 25°C (in the

nasal cavity), but very poorly at higher temperatures in the human body (such as the lower respiratory tract)[11]. In the United States, the effectiveness of LAIV in comparison to inactivated vaccines was quite low in subjects aged 2–17 years during influenza seasons 2013–2014 and 2015–2016, especially against influenza A/H1N1pdm09[12,13]. Therefore, the CDC's Advisory Committee on Immunization Practices (ACIP) decided not to recommend LAIV for use during the 2016–2017 influenza season[14]. Flublok® is a third type of human influenza vaccines. This vaccine is a trivalent, seasonal influenza vaccine consisting of three full-length recombinant hemagglutinin influenza virus proteins. The vaccine is produced in an insect cell line derived from Sf9 cells of the fall armyworm, *Spodoptera frugiperda*. Each of the three HAs is expressed using a viral vector (baculovirus *Autographa californica* Nuclear Polyhedrosis Virus) that is non-pathogenic for humans.

However, these vaccines do not elicit a strong heterosubtypic immune response, since the majority of the vaccine-induced antibodies fail to cross-react with hetero(sub)typic HA and NA, and if cross-reactive T cell responses are induced, these responses are much lower than the homologous T cell response[15,16]. It was shown that there were no increases in the mean levels of influenza A virus-reactive IFN-γ^+ T cells and NK cells in adults given either LAIV or TIV while LAIV did have a positive effect on influenza A virus-specific IFN-γ^+ $CD4^+$ and $CD8^+$ T cells in children aged 5–9 years[17]. Additionally, TIV treatment had a significant effect in 6-month to 4-year-old children on the level of influenza A virus-reactive T cells; LAIV was not evaluated in this age group. This indicates that the efficacy of inducing a cellular immune response of currently used vaccines is highly dependent on age, type of vaccine, and prevaccination levels of immune reactivity to influenza A virus[17]. In young children, who are often immunologically naïve to influenza virus, inactivated vaccines may even hamper the induction of cell-mediated immunity that would be otherwise induced by natural (disease causing) infections[18]. Hence, the big challenge in influenza vaccine development remains the induction of broadly neutralizing antibodies and long-lasting heterosubtypic cellular immune responses.

3.2 Immune response to influenza virus infection

3.2.1 Innate immunity

3.2.1.1 Extracellular barriers to overcome

Before it can infect respiratory epithelial cells, the influenza virus has to cross or circumvent two main barriers. The first barrier is the mucus layer that lines the respiratory tract. This layer forms a physical barrier consisting of a mixture of cells, cellular debris and polypeptides, held together by macromolecular constituents called mucins. Mucins are a family of glycoproteins that are secreted or remain membrane associated. They are heavily

glycosylated, and the terminal sialic acid residues of these glycans are linked to galactose. It has been shown that upon viral infection of the respiratory tract, the production of mucus in the epithelial surfaces of the respiratory tract increases[19,20]. To cross this mucus layer, influenza viruses rely on the enzymatic activity of NA, which cleaves off terminal sialic acids from glycans[21].

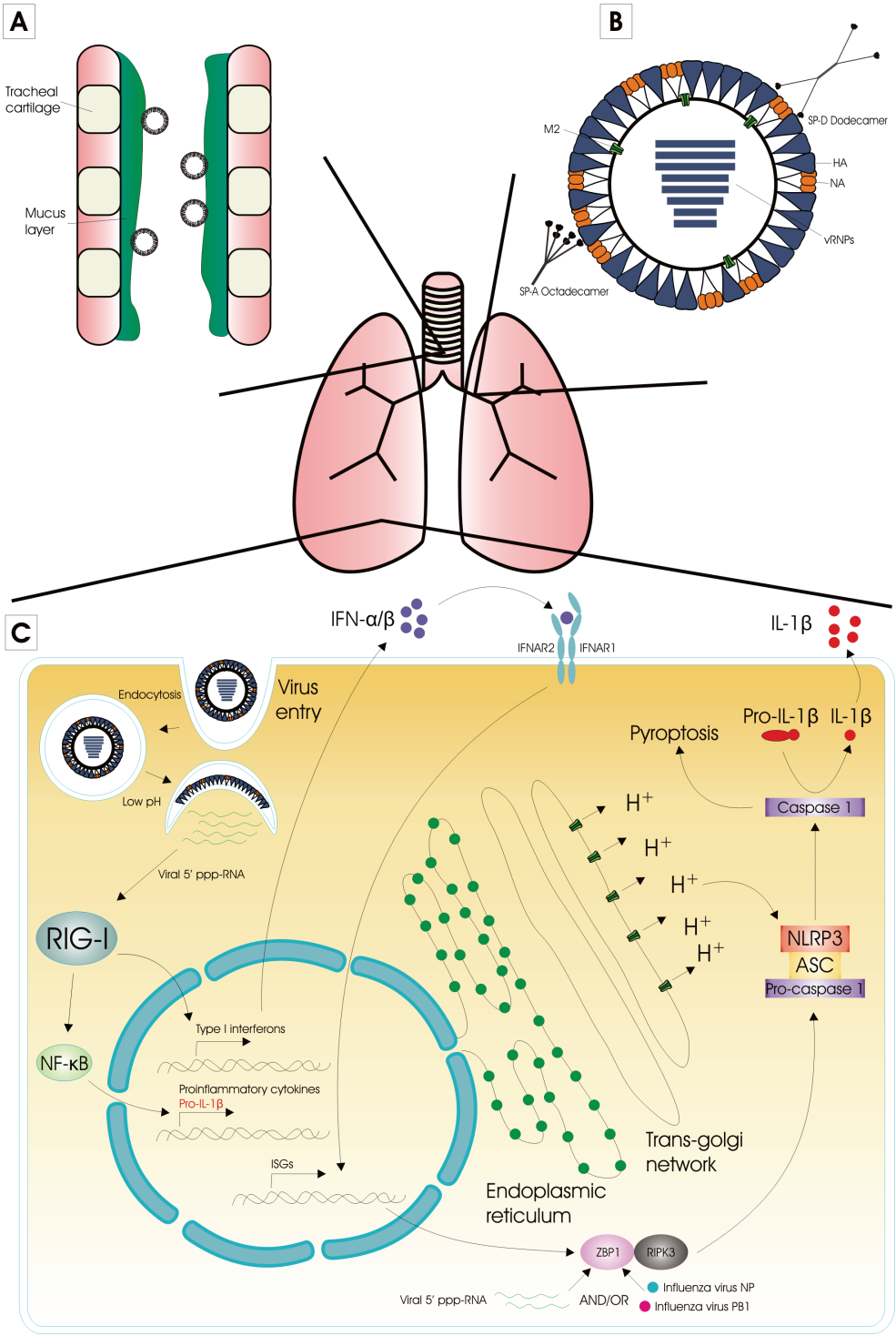

Figure 3.1 Innate immunity against influenza virus infection. (a) The first barrier that the influenza virus has to overcome, is the mucus layer that lines the respiratory tract. To cross this barrier, influenza viruses rely on the enzymatic activity of the neuraminidase glycoprotein; **(b)** The second barrier consists of carbohydrate-binding proteins called lectins. Surfactant proteins A (SP-A) and D (SP-D) are the main two lectins involved in anti-influenza activity. SP-A acts as a decoy receptor for influenza virus, and SP-D binds to oligosaccharides on influenza hemagglutinin (HA) and neuraminidase (NA) proteins; **(c)** Once influenza virions have reached respiratory epithelial cells they recognize sialic acid-containing host cell receptors by the HA glycoprotein. This is followed by endocytosis of the influenza virus and the virion particle ends up in the early endosomes. After acidification of the endosome and subsequent membrane fusion, the genomic RNA segments of the influenza virus are released into the cytosol. The two major PRRs that are responsible for the cytoplasmic sensing of influenza virus infection are retinoic acid inducible gene-I (RIG-I) and NOD-like receptor family pyrin domain containing 3 (NLRP3) protein. Activation of RIG-I—by interaction with 5′ triphosphorylated RNA (5′ ppp-RNA)—results in the production of proinflammatory cytokines and type I interferons (IFNs), which in turn induce the expression of interferon-stimulated genes (ISGs). NLRP3 is a part of the NLRP3 inflammasome, which becomes activated by the proton gating activity of influenza A virus M2. An additional way to activate the NLRP3 inflammasome is through the interferon-stimulated gene *Z-DNA binding protein 1* (*ZBP1*). ZBP1 interacts with receptor-interacting serine/threonine-protein kinase 3 (RIPK3), and this ZBP1-RIPK3 complex is responsible for the activation of the NLRP3 inflammasome (cfr. Paragraph 2.7.9). This leads to the conversion of pro-IL-1β to IL-1β, which is a proinflammatory cytokine that is involved in the induction of Th17 cells and the expansion of $CD4^+$ T cells. Active caspase-1 can also induce an inflammatory form of cell death called pyroptosis.

The second barrier consists of proteins that bind to specific carbohydrate structures, so-called lectins. In the lung, the two main lectins involved in anti-influenza activity are surfactant proteins A (SP-A) and D (SP-D). These lectins hamper influenza virus infection by different mechanisms. SP-A is sialylated and therefore acts as a decoy receptor for influenza virus (γ-inhibition)[22], while SP-D binds mannose-rich oligosaccharides on influenza virus HA and NA proteins (β-inhibition) (Figure 3.1)[23].

3.2.1.2 Sensing of influenza virus infection

Once influenza virions have crossed the mucin- and lectin-rich layer that lines the respiratory tract, they reach respiratory epithelial cells. After recognition of the sialic acid-containing host cell receptors by the HA glycoprotein, endocytosis of the influenza virus is triggered and the virion particle ends up in the early endosomes. The passage in the endosomes allows entry of protons and at a later stage potassium ions into the virions which primes them for genome delivery. Matrix protein 2 (M2) fulfills an important function in this process[24]. The interior pH of the endosome becomes acidic, which induces a conformational change in the HA protein. This leads to the insertion of the fusion peptide of HA into the host membrane, and formation of a fusion pore. This pore allows the release of the genomic RNA segments of the influenza virus into the cytosol[25].

The two major pattern recognition receptors (PRRs) that are responsible for the cytoplasmic sensing of influenza virus infection are retinoic acid inducible gene-I (RIG-I) and NOD-like receptor family pyrin domain containing 3 (NLRP3) protein (Figure 3.1)[26,27].

Activation of RIG-I by interaction with 5′ triphosphorylated RNA, results in the production of proinflammatory cytokines and type I interferons (IFNs), which in turn induce the expression of interferon-stimulated genes (ISGs) via the JAK/STAT signaling pathway[28]. One of the most important antiviral ISGs is the *myxovirus* (*Mx*) gene, which encodes the MxA and Mx1 protein in human and mouse, respectively. Mx proteins are dynamin-like GTPases with strong and broad antiviral activity[29]. Type I IFNs also modulate the adaptive immune response: they form a bridge between innate and adaptive immunity by stimulating dendritic cells (DCs), which results in enhanced antigen presentation to $CD4^+$ and $CD8^+$ T

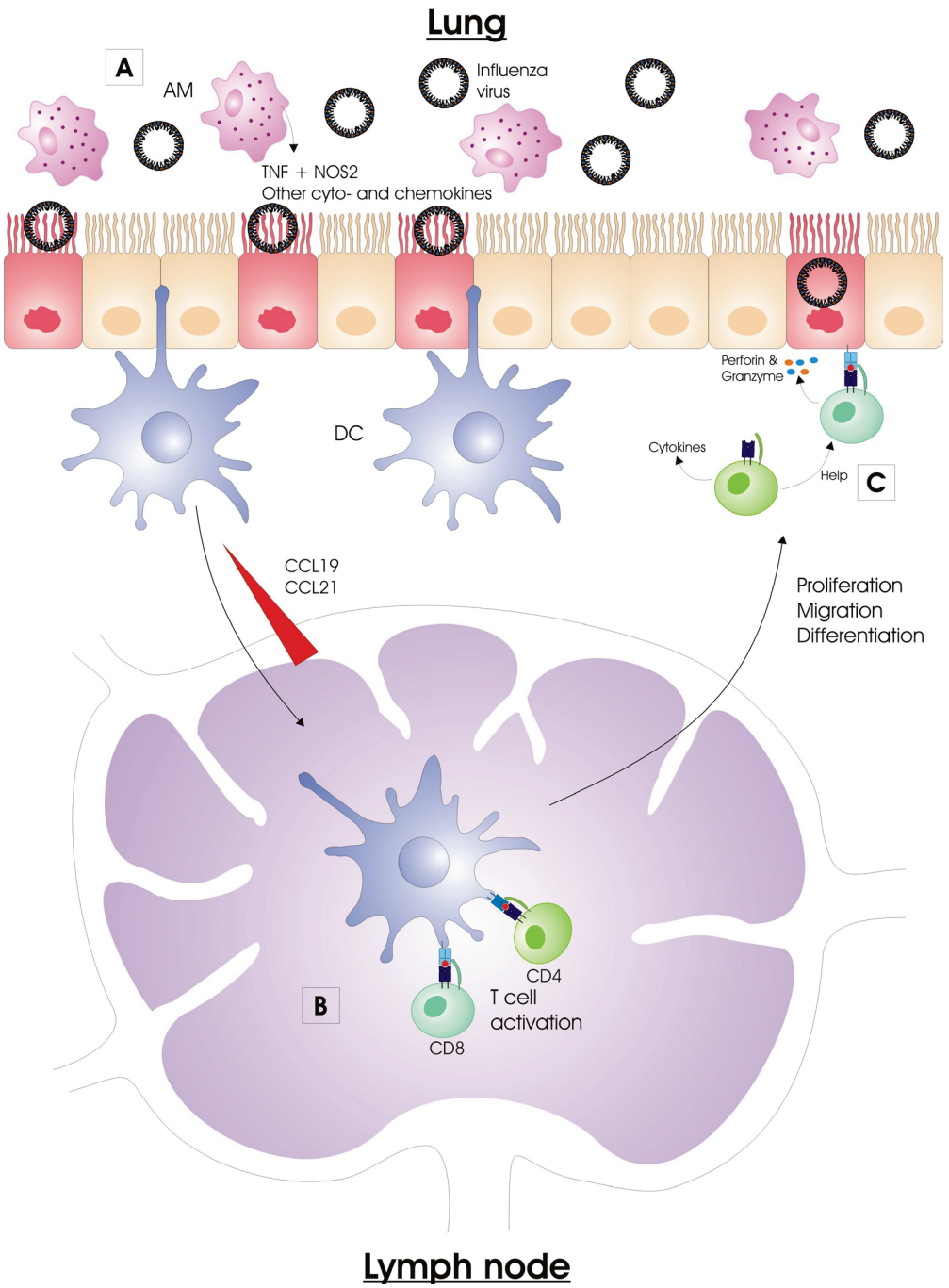

Figure 3.2 Adaptive immunity against influenza virus infection. (a) Alveolar macrophages (AMs) reside in the alveolar lumen and are considered the first immune cell type to encounter respiratory pathogens. Activated AMs produce nitric oxide synthase 2 (NOS2) and tumor necrosis factor (TNF), by which they also contribute to lung pathology upon influenza virus infection; **(b)** Dendritic cells (DCs) are professional antigen-presenting cells (APCs). The conventional DCs (cDCs), which are located underneath the airway epithelium barrier and above the basal membrane, continuously monitor the airway lumen with their dendrites. Once the cDCs acquired viral antigen, they migrate to the draining lymph nodes (DLNs) via a CCL19 and CCL21 chemokine gradient along the afferent lymphatic system. In the LNs, DCs present processed antigen to naïve T cells. The DC degrades viral proteins, and the resulting peptides (antigens) are presented by MHC class I or II proteins for specific $CD8^+$ and $CD4^+$ T cells respectively. The T cells that are specific for the antigen become activated and expand clonally. The newly activated T cells begin to acquire effector functions, and migrate from the LNs to the site of infection; **(c)** $CD8^+$ T cells can kill infected respiratory epithelial cells, and, by this, clear the virus from the lungs. This is done by exocytosis of granules that contain perforins and granzymes. $CD4^+$ T cells on the other hand mainly provide help to other immune cells, and regulate the immune response by producing a vast array of cytokines.

cells[30]. NLRP3 is a part of the NLRP3 inflammasome, which becomes activated by the proton gating activity of influenza A virus M2[31]. This leads to the activation of caspase-1 and conversion of pro-IL-1β to IL-1β, which is a proinflammatory cytokine that is involved in the induction of T helper 17 cells and the expansion of $CD4^+$ T cells[32,33].

3.2.1.3 Alveolar macrophages

Alveolar macrophages (AMs) reside in the alveolar lumen and are considered the first immune cell type to encounter respiratory pathogens. When activated, AMs phagocytose virus particles and (apoptotic) infected cells, and consequently limit viral spread[34,35]. Activated alveolar macrophages also produce nitric oxide synthase 2 (NOS2) and tumor necrosis factor (TNF), by which they also contribute to lung pathology upon influenza virus infection (Figure 3.2)[36,37]. In addition, AMs regulate the adaptive immune responses. Depletion of AMs prior to influenza virus infection increases the primary cytotoxic T cell response in mice[38]. In pigs, conversely, depletion of AMs prior to influenza virus infection reduces the antibody titers and the number of virus-specific cytotoxic T cells. This effect was probably caused by an alteration of the expression pattern of inflammatory cytokines, since pro-inflammatory cytokines TNF and IFNγ are downregulated in the lung in contrast to the anti-inflammatory cytokine IL-10, which is upregulated in these animals after AM-depletion and subsequent influenza virus infection[34]. The downregulation of pro-inflammatory cytokines is also observed in mice infected with a reassorted H1N1 virus containing the 1918 Spanish flu HA and NA after AM-depletion[35]. AMs are very important for certain adaptive immune responses against influenza viruses. For instance, our group previously showed that AMs are essential for protection by anti-M2e IgG[39].

3.2.1.4 Dendritic cells

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) which form an important link between the innate and adaptive immune systems. DCs can be considered the sentinels of the vertebrate immune system at body surfaces. In the lung, DCs perform multiple tasks, such as recognition and acquisition of antigens derived from pathogens and allergens, antigen transportation to the draining lymph nodes and induction of $CD4^+$ and $CD8^+$ T cell immunity[40]. The conventional DCs (cDCs) are located underneath the airway epithelium barrier and above the basal membrane. They continuously monitor the airway lumen with their dendrites, which they can extend into the airway lumen through the tight junctions of the epithelial cell layer[41]. During an infection, DCs will initiate the adaptive immune response by presenting viral antigens to B and T lymphocytes in the draining lymph nodes (DLNs)[42,43].

3.2.1.5 Natural killer cells

Natural killer cells (NKs) are cytotoxic effector cells of the innate immune system. They can lyse influenza virus-infected cells following direct or indirect recognition of such target cells. The natural propensity of the cytotoxicity receptors NKp44 and NKp46 to recognize HA on the surface of infected cells contributes to a direct elimination of such cells[44,45]. Indirect recognition and subsequent lysis of infected cells by NKs is mediated by immune complexes at the surface of IgG-opsonized influenza virus-infected cells in a process called antibody-dependent cell cytotoxicity (ADCC)[46].

3.2.2 Adaptive immunity

3.2.2.1 Activation of antigen-presenting cells

DCs can be considered the most important APCs in the lungs. In the steady state lung, several distinct DC subsets are present. These subsets differ in phenotype, anatomic location, and function. The $CD103^+$ and the $CD11b^{hi}$ DC subsets, located intraepithelial and submucosal/interstitial, respectively, are the main DC populations in the lungs and are collectively often referred to as cDCs[47].

After activation, respiratory cDCs have to acquire antigen derived from influenza virus for delivery to the DLN. The acquisition of antigen by DCs occurs via several mechanisms (Figure 3.2). The most likely way is by direct infection of the DCs, but different DC subsets differ in susceptibility to influenza virus infection[48]. Such an infection is probably abortive, so DCs fail to release infectious virions, but acquire sufficient amounts of viral antigens that are processed and uploaded into their MHC-I and -II molecules[49]. Another mechanism for cDCs to acquire viral antigen is by phagocytic engulfment of cell-free virions or dying/dead

infected cells that harbor viral antigen. Lastly, antigen-acquisition can also occur through a membrane nibbling process, called trogocytosis[50-52].

3.2.2.2 Antigen presentation

Once the cDCs acquired viral antigen, they migrate to the draining lymph nodes via the afferent lymphatic system. This migration occurs along a CCL19 and CCL21 chemokine gradient, and is dependent on expression of the chemokine receptor CCR7[53]. Although there are other APCs present in the DLN (such as plasmacytoid DCs, monocyte-derived DCs and macrophages), cDCs serve as the primary APCs for naïve T cells[54]. $CD103^+$ DCs are the most potent APCs for the activation of cytotoxic T lymphocytes (CTLs) after influenza virus infection. These DCs can engulf influenza virus infected cells, and process and present virus antigen from these cells to CTLs, a process that is known as cross-presentation. $CD103^+$ DCs acquire an antiviral state, which is induced by type I IFN signaling and characterized by the elevated mRNA-levels of IFN-inducible genes such as *ISG 15*, *OAS1a*, *Mx1*, *Ifitm1*, *Ifitm3* and *PKR*[55]. $CD103^+$ DCs represent the first wave of migration and antigen presentation to CTLs. Afterwards, influenza virus antigen is replenished by blood-derived $CD11b^{hi}$ DCs that migrate to DLNs from the lung[56,57]. This is thought to serve as a form of amplification loop for the generation of CTLs. Both $CD103^+$ and $CD11b^{hi}$ DCs also activate naïve $CD4^+$ T cells with equal efficiency[58].

Naïve T cells circulate between blood and lymph nodes, where they remain for 12–24 h before returning to the blood and visiting the next lymph node. In the LNs, DCs present processed antigen to these naïve T cells. The DC degrades viral proteins, and the resulting peptides (antigens) are presented by MHC class I or II proteins. MHC class I/antigen complexes are presented on the cell membrane for recognition by specific $CD8^+$ cytotoxic T cells. MHC class II presentation of the antigen on the cell membrane is recognized by $CD4^+$ T helper (Th) cells. The T cells that are specific for the antigen—*i.e.*, the T cells with a matching T cell receptor (TCR)—become activated and expand clonally. This expansion is extensive, as one naïve T cell can give rise to tens of thousands of progeny T cells[59]. The freshly activated T cells begin to acquire effector functions, such as the ability to produce effector cytokines. For activated $CD4^+$ T cells, the cytokine environment is very important to differentiate into different effector Th cell types[60].

3.2.2.3 Lymphocyte migration to the infected lung

In order to migrate from the LNs to peripheral tissues, activated T cells change the expression profile of homing molecules. Mature naïve T cells express lymphoid homing receptors CD62L and CCR7, which are necessary for migration to secondary LNs[61,62]. Once these T cells are activated after a DC encounter, they migrate to the site of infection. In

order to get out of the LNs, downregulation of CD62L and CCR7, and upregulation of other receptors is necessary. It has already been shown that different T cell subsets express their own specific chemokine receptor repertoire after activation, which allows them to be recruited to different peripheral tissues[63]. Recruitment of activated T cells to the infected lung occurs via nonspecific and specific routes. CD11a, which is a subunit of the integrin lymphocyte function-associated antigen-1 (LFA-1), is responsible for the nonspecific recruitment of activated T cells into the lungs, because this protein is upregulated in activated T cells, and its ligand ICAM-1 (Intercellular adhesion molecule-1) is expressed in peripheral tissues[64]. Specific recruitment of activated T cells is more complicated and less well understood. Mikhak and colleagues observed that lung DCs are responsible for the upregulation of chemokine receptor CCR4 on effector T cells, which allows for selective recruitment into the infected lung, where CCL2, CCL3, CCL5, CCL17 and CCL22, the ligands of CCR4, are produced[65]. However, it seems that this is not the case for $CD8^+$ T cells, or at least that other mechanisms cannot be excluded. For this T cell population multiple recruitment mechanisms are implied to get the cells to the lung interstitium. Galkina and colleagues showed that migration of effector $CD8^+$ T cells is promoted by expression of the chemokine CCL5 in the lung interstitium[66]. Slütter *et al.* on the other hand, have indicated that expression of CXCR3 on antigen-specific memory $CD8^+$ T cells, from vaccinated mice, is critical for their migration to the airways[67]. Then again, Lim and coworkers recently reported the importance of the chemokine CXCL12 which is mainly produced by neutrophils, for virus-specific recruitment of $CD8^+$ T cells and antiviral effector functions[68].

3.2.2.4 Viral clearance

Once influenza-specific effector T cells have entered the respiratory tract, there is a significant impact on viral titers through the expression of cytokines (IFNγ, TNF, IL-4 and IL-10) and direct lysis of infected cells. $CD8^+$ cytotoxic T cells mainly contribute to viral clearance through the cytotoxic elimination of influenza virus-infected respiratory epithelial cells. Such elimination is possible by two mechanisms, *i.e.* the release of perforins and granzymes by the activated T cell or the engagement of tumor necrosis factor (TNF) family members on the surface of target cells with their ligands. Both mechanisms result in apoptosis of the target cell[69]. Influenza-specific $CD4^+$ Th cells act directly and indirectly on the viral clearance process. Primarily they act indirectly by producing cytokines and helping B cells and $CD8^+$ T cells[70-72]. Th cells can also directly eliminate infected cells, but this mechanism of action is rather accessory to their function as helper cells[73].

3.3 T cell response to influenza virus infection

3.3.1 Primary T cell response: Deflowering the T cells

Once infected with influenza virus, the epithelial cells start producing inflammatory cytokines. The first cytokines are IFNα, TNF, IL-1α and IL-1β, followed by IL-6, IL-8, monocyte chemoattractant proteins (MCPs), and macrophage inflammatory proteins (MIPs). Several of these cytokines have a chemotactic function, and thus attract innate immune effectors and antigen presenting cells to the site of infection. DCs that got recruited this way, take up viral particles and influenza-derived antigens. This triggers DC activation, maturation and migration to the draining LNs.

3.3.1.1 CD4⁺ T cell primary responses

Once naïve CD4⁺ T cells have matured, they traffic from the thymus to the secondary lymphoid tissues. Here the influenza-specific CD4⁺ T cells interact with mature DCs who carry viral antigens, and get activated. The activated T cells start proliferating and acquire effector functions. Activated CD4⁺ T cells differentiate to different subsets (Th1, Th2, Th17, Tfh, Treg), which can be characterized by their own distinct cytokine pattern. This differentiation is primarily promoted by the cytokine environment in which they are formed[74]. T helper (Th) 1 cells mainly produce IFNγ, TNF and IL-2. In contrast, Th2 cells do not produce IFNγ, but they do produce large amounts of IL-4, IL-5 and IL-13. The main function of Th1 cells is enhancing the pro-inflammatory cellular immunity[75,76], while Th2 cells promote non-inflammatory immune responses and can induce the production of most classes of antibodies, mainly IgG1 and IgE[77]. Influenza virus infection generates both a Th1 response, where CD4⁺ T cells produce IFNγ, TNF and IL-2[71,78], as well as a Th2 response, which helps formation of a robust antibody response[79]. However, following influenza virus infection, the CD4⁺ T cell response is biased toward a Th1 effector response[80].

Next to Th1 and Th2 cells, there is a third major CD4⁺ T cell subpopulation called Th17 cells[81]. These cells are characterized by the production of IL-17 and IL-22[82]. They play an important role in the protection against opportunistic bacterial pathogens, such as is seen after influenza virus infection. Influenza A virus, however, employs mechanisms that inhibit a strong Th17 response, leading to an increased susceptibility to secondary bacterial infections[83]. The direct role of Th17 cells in the immune response against influenza virus is not totally clear yet, with studies pointing to both a possible beneficial or detrimental effect of a Th17 response. On the one hand, it has been shown that IL-17 can protect mice against a lethal infection with influenza A/Puerto Rico/8/34 (H1N1) and A/Alaska/6/77 (H3N2), and that it has a critical role in recruiting B cells to the pulmonary site of infection. Additionally, the IL-17 response contributes to a better survival percentage after a lethal

influenza A/Puerto Rico/8/34 infection[84,85]. However, other studies indicate that IL-17 and the Th17 response may contribute to severe pulmonary immunopathology after influenza virus infection[81,86], and pre-induction of the Th17 immune response results in an exacerbation of lung pathology after influenza virus infection[87]. However, only the latter study by Gopal and coworkers investigated the direct *in vivo* effect of Th17 cells on lung pathology in an influenza virus infection model. The two former studies investigate the effect of IL-17 during influenza virus infection, but use a different infection model[85,86]. The remaining studies make use of *in vitro* differentiated $CD4^+$ and/or $CD8^+$ T cells which produce IL-17, but cannot be compared because of the difference in research question and models used[81,84].

A fourth $CD4^+$ T cell subset comprises the regulatory T cells (Tregs), which contribute to homeostasis of the immune system and tolerance to self-antigens. This subset on itself can be further subdivided into two groups, natural Tregs (nTregs) that are generated in the thymus by MHC-II-dependent T cell receptor (TCR) interactions[88], and induced Tregs (iTregs) which are generated in the periphery during an immune response[89]. Influenza virus infection generally triggers a robust Treg response, where iTregs suppress antigen-specific $CD4^+$ and $CD8^+$ T cell proliferation and cytokine production in an antigen-dependent matter[90].

The last $CD4^+$ T cell subset discussed here is made up by the follicular helper T cells (Tfh). These cells are paramount in the formation and function of germinal centers, which are located in secondary lymphoid organs, and are the primary sites of B cell affinity maturation[91]. Here, the Tfhs play a pivotal role in providing help signals to B cells, which are essential for their survival and proliferation. After somatic hypermutation, the highest affinity B cells are selected by Tfhs for another round of proliferation and mutation[92,93]. Most human vaccines work on basis of long term protective antibody responses, so Tfhs are probably mediators of development of protective immunity by vaccines. In fact, it has already been shown that Tfhs are a limiting factor for generating antibody responses after immunization[94]. Learning more about this $CD4^+$ T cell subset might be essential for the further improvement of human vaccination.

3.3.1.2 $CD8^+$ T cell primary responses

Naïve $CD8^+$ T cells get activated in the draining lymph nodes after recognition of a viral epitope-MHC-I complex on an antigen presenting cell. This interaction initiates the differentiation of naïve $CD8^+$ T cells into mature CTLs. CTLs then migrate to the site of infection, which in the case of an influenza virus infection is the infected lung[95]. There, their main task is killing infected respiratory epithelial cells, and by this, clearing the virus from

the lungs. To kill infected cells, CTLs use two distinct cytotoxic mechanisms: (1) granule exocytosis; and (2) the engagement of tumor necrosis factor (TNF) family members with their respective ligands. Granule exocytosis refers to the lysosomes that are released by CTLs after they interacted with influenza virus antigen-derived peptides complexed with MHC-I on the infected cell through their T cell receptor (TCR). These granules contain the pore-forming protein perforin and serine proteases called granzymes. Perforin forms pores in the target cell membrane, which allows the pro-apoptotic granzymes to enter the cells and initiate programmed cell death. Cytotoxicity can also be mediated by several members of the TNF family, with FasL being the most important example of these family members. TCR engagement of peptide/MHC-I-complexes results in upregulation of FasL and its migration to the cell membrane of CTLs. Fas proteins are expressed on the cell membranes of most cell types, and these proteins get oligomerized by interacting with their ligand FasL. This oligomerization triggers an apoptotic cascade in the target cell, resulting in programmed cell death[96]. Another cytotoxicity-inducing TNF family member is the TNF-related apoptosis-inducing ligand (TRAIL). TRAIL is comparable to FasL, in that interaction with its ligand (TRAIL-death receptor) also initiates an apoptotic pathway in the target cell. Evidence for its role in protection against influenza virus was reported by Brincks *et al.* who showed that TRAIL-deficiency decreases $CD8^+$ T cell-mediated cytotoxicity, leading to more severe influenza virus infections[97].

3.3.2 Memory T cell response: The T cells remember

After clearance of the primary infection in the lungs, the effector T cells go into a contraction phase. Herein, 90%–95% of all antigen-specific T cells undergo apoptosis. The small portion of cells that remains are destined to be long-living memory T cells[98,99]. How this transition takes place, and how the cell-fate of a T cell is decided, is still unclear. Several models have been proposed and are reviewed by Buchholz *et al.*[100]. What is known for sure, is that the formation and homeostasis of memory $CD4^+$ and $CD8^+$ T cells is dependent on IL-7 and IL-15[101-105]. Upon reinfection by the same pathogen, memory T cells begin producing effector molecules, undergo a massive clonal expansion, and differentiate to secondary effector T cells in order to control the infection faster. Some key features of memory T cells—which makes them able to respond faster than 'normal' effector T cells—are a high proliferative potential, a multipotent state, long term survival and self-renewal in absence of antigen (in presence of IL-7 and IL-15)[106].

The past fifteen years memory T cells have been subject of many studies, which led to the current "classification" of memory T cells. For a long time it was thought that memory T cells could be divided into two groups, the high CCR7- and CD62L-expressing central memory T cells (Tcm) and low CCR7- and CD62L-expressing effector memory T cells (Tem).

The first group of memory T cells patrols secondary lymphoid organs like naïve T cells do, but upon antigen-recognition they undergo a rapid and robust proliferation, differentiation and migration to the site of infection. The latter group recirculates between blood and non-lymphoid tissues[107]. Upon antigen-recognition they rapidly execute their effector functions like freshly stimulated effector T cells would do[107]. More recently, a third memory population was defined: tissue-resident memory T cells (Trm), which are derived from precursors that entered the tissue during the immune effector phase and remained in this tissue[108,109]. Instead of CCR7 and CD62L, Trm-specific markers are the glycoprotein CD69 and the integrin CD103[110-112], although the expression of the latter is more predominant on $CD8^+$ Trm cells than on $CD4^+$ Trm cells[108]. $CD4^+$ Trm cells are better associated with expression of CD69 and CD11a[109,113].

3.3.2.1 $CD4^+$ memory T cells

$CD4^+$ memory T cells have long been studied less intensely than $CD8^+$ memory T cells. The main reason for this is that $CD4^+$ memory T cells do not expand as exuberantly as $CD8^+$ memory T cells, and consequently are not present in large numbers after re-exposure to antigen[114]. For respiratory viruses, $CD4^+$ Trm cells seem to be important for optimal protection against reinfection[109,115]. For influenza virus, it has been shown that $CD4^+$ T cell epitopes are conserved within different subtypes of influenza virus. Interestingly, in people infected with seasonal influenza virus, virus-specific $CD4^+$ T cells have been isolated which cross-react with emerging reassortant strains like H5N1[116]. Alexander and colleagues have shown that DNA vaccines, containing several $CD4^+$ T cell epitopes, protected against lethal influenza virus infection[117]. Next to the circumstantial evidence, recent studies have elucidated a lot more about the role of $CD4^+$ memory T cells in the immune response to influenza virus infection. It has been shown that $CD4^+$ memory T cells can direct influenza virus clearance in absence of B cells and $CD8^+$ T cells, however they cannot provide full protection[118]. In cooperation with other cell types, however, $CD4^+$ memory T cells can provide clear protection during re-infection[119-121]. These findings have great significance for the production of universal influenza vaccines that aim at inducing long lived T cell responses.

3.3.2.2 $CD8^+$ memory T cells

Memory $CD8^+$ T cells, like $CD4^+$ memory T cells, have the ability to rapidly generate effector functions. They also produce a burst of secondary CTLs that can rapidly contain secondary infections. Repetitive reactivation of the memory $CD8^+$ T cells, either through booster vaccinations or successive infections, augments the effector-like properties of memory $CD8^+$ T cells and the frequency of Tem cells in the resulting memory T cell pool[122]. The importance of memory $CD8^+$ T cells has already been illustrated in humans. People without

detectable pre-existing antibodies to the 2009 pandemic H1N1 strain were monitored following the global spread of this virus. From this it was evident that people that showed no or minor disease symptoms had higher levels of pre-existing FLUAV-specific $CD8^+$ Tem cells. This study also showed no clear correlation between disease severity and pre-existing memory $CD4^+$ T cells[123]. This is rather striking because Wilkinson and colleagues noticed an inverse correlation between the presence of pre-existing $CD4^+$ memory T cells and disease severity following a controlled challenge[124]. The reason for these different observations is currently unclear.

$CD8^+$ Trm cells form the frontline against secondary infection by influenza viruses in the lung compartment. Upon reinfection, they can immediately acquire effector functions. How come that these $CD8^+$ Trm cells are not infected by influenza viruses, which, after all, merely require a sialic acid-containing receptor on a mammalian cell for their entry? It has been reported that $CD8^+$ Trm cells that are formed after an influenza virus infection, show a massive upregulation of the interferon-induced transmembrane protein 3 (IFITM3)[125]. The same observation was made in $CD8^+$ Trm cells from the brain after infection with Vesicular Stomatitis Virus[126]. IFITM3 is a potent antiviral molecule that can render resistance to infection with influenza virus by interfering with the fusion of the influenza virus envelope and the late endosome cell membrane, more specifically by blocking the formation of fusion pores[127]. All these observations make it very clear that generating memory T cells should be one of the most important objectives in the development of potent anti-influenza vaccines.

3.4 Vaccines

3.4.1 Current influenza vaccines

Annual influenza vaccination is the most effective way to prevent influenza virus infection[8], and is generally done with inactivated (TIV or QIV) or live-attenuated vaccines (LAIV). TIV induces higher titers of serum hemagglutination-inhibiting (HI) IgG and IgA antibodies than LAIV[128]. LAIV in its turn induces higher levels of nasal-wash IgA than TIV which mainly elicits IgG in the upper respiratory tract mucosa[129,130]. Inducing efficient cellular immune responses in the lung is dependent on efficient replication of the virus in the lung. TIV does not elicit cellular immunity because it is inactivated, and LAIV is designed in such a way that it only replicates in the upper respiratory tract. Therefore, both vaccines do not show much potential for inducing efficient cellular immune responses. However, cellular immunity was demonstrated in the lung after LAIV-administration in animal models[131]. Additionally, in humans, there is also an indication that LAIV elicits cellular immunity[17].

In the past, LAIV seemed to be superior to TIV in reducing laboratory-confirmed influenza in children[132]. Consequently, the use of LAIV was recommended in the UK, Germany, Israel,

Canada and Sweden for children of various ages[133], and in the USA for children aged two to eight years[134]. In adults, the efficacy of LAIV is somewhat debated. In a study conducted by Monto *et al.*, LAIV was 50% less efficient than TIV in the reduction of laboratory-confirmed influenza during the 2008–2009 influenza season[135]. Nichol and colleagues showed less efficacy in subjects between the age of 50 and 64, which led to the disapproval of LAIV for this age group in the USA[136]. Other studies show that LAIV reduces illness by 85% compared to TIV (subjects aged 18–41 years), and that LAIV does protect against influenza virus infection in adults above the age of 60 years[128,137]. However, as mentioned earlier, use of LAIV is no longer recommended for the upcoming 2016–2017 season because of its poor effectiveness against the H1N1 pandemic of 2009[12,13].

3.4.2 T cells in heterologous protection against influenza viruses

Current vaccines are mainly designed to elicit humoral rather than cellular immunity. The protective cut-off for evaluating vaccines is hence based on a hemagglutination-inhibition (HI) titer of 1/40 or more. This cut-off already dates from 1972, when Hobson and colleagues showed that a HI-titer of 1/36 corresponded with a 50% reduction of infection rate[138]. However, the 1/40 HI-titer correlate of protection is not a good cut-off for evaluating the vaccine-efficacy in children and elderly people[139,140]. Alternatively, a new correlate of infection should be developed, which takes into account the HI-titer and the influenza-specific cellular immune response. A vast amount of epidemiological studies supports this statement. During the 1977 influenza season, protection against homologous H1N1 virus infection was illustrated when H1N1 re-emerged after 20 years. Individuals born before 1955 had a lower influenza A/H1N1 attack rate in 1977 than people born after 1955[141]. A surveillance study performed during more than 10 years in families in Cleveland showed that adults who were repeatedly infected with influenza virus before the pandemic year 1957 (H2N2), had a much lower incidence of influenza during the pandemic year[142]. Re-analysis of this study provided indirect proof that cellular immunity is involved in long-lived protection against influenza virus infection[143]. Studies performed during and after the 2009 Mexican flu pandemic showed comparable results[123,144]. In an experimental challenge study performed by McMichael *et al.*, a direct correlation between pre-infection levels of CTLs and virus clearance was detected[145]. Similarly, in another controlled infection experiment, a strong protective role for memory $CD4^+$ T cells was elucidated[124]. These studies were the starting point for the mounting evidence of the role of T cells in protection against influenza virus.

3.4.3 Vaccination-induced T cell immunity: To serve and protect

People that got infected with influenza virus in the past, have a two-part defense to neutralize a homologous reinfection. The first line of defense consists of antigen-specific

antibodies that were elicited by the previous influenza virus infection. This is considered as neutralizing immunity, because the antibodies can directly attack the virus particles. However, often not all of the inoculum is neutralized or opsonized by the antibodies, which leaves some viral particles able to infect host cells. This is where the cellular immune response also kicks in. Vaccination strategies should induce both lines of the defense mechanism, and ideally in such a way that there is broad heterologous protection against subsequent influenza virus infections. As mentioned before, current inactivated vaccines do not elicit a strong T cell response. However, this might, in part, be overcome by the use of the right adjuvants. Adjuvants help to determine the type and size of the immune response provoked by vaccination. They can act as a depot and stabilizer for the vaccine, and stimulate innate immune responses, affecting the subsequent adaptive immune responses. The only adjuvants approved for use in humans are aluminum salts (aluminum hydroxide, aluminum phosphate and aluminum sulphate) and squalene-based adjuvants (AS03 and MF59); these adjuvants have been shown to promote induction of T cell responses[146]. Inactivated vaccines elicit neutralizing antibodies, which could impair the induction of cross-reactive cellular responses. This is of special concern for young children who are in general immunologically naïve for influenza virus[18]. This problem can be avoided by inducing infection-permissive immunity, which may still be protective, but allows virus-induced cross-reactive immune responses. A vaccine which targets the conserved ectodomain of influenza virus matrix protein 2 (M2e) delivers this kind of non-neutralizing immunity, since anti-M2e antibodies rely on Fc receptors and innate immune components to provide protection[39]. Another promising vaccine candidate targets the influenza virus neuraminidase (NA) glycoprotein. Antibodies that inhibit NA-activity do not block entry of the virus into the host cell, so such antibodies do not provide sterilizing immunity and may contribute to immunity against a virus that has a similar NA type[147].

Last years, recombinant T cell-inducing vaccines are getting into play, the most advanced vaccine candidate is based on Modified Vaccinia Ankara (MVA) viruses expressing influenza virus NP and M1 antigens[148]. Individuals vaccinated with this MVA–NP+M1 show an increase in IFNγ-producing (cytotoxic) $CD8^+$ T cells, and consequently an increase in protection against influenza virus infection[149,150]. Coadministration of this MVA-based vaccine with the seasonal TIV results in an increase of influenza strain-specific antibody responses and a boost of memory T cells capable of recognizing a range of influenza A subtypes[151].

FLU-v is a new vaccine candidate which consists of four peptides (three peptides derived from influenza A virus (FLUAV), and one peptide from influenza B virus. These peptides are synthetic multi-epitope peptides that were identified *in silico*[152]. The vaccine is already

proven to be safe and well-tolerated in phase I clinical trials[153]. Peripheral blood mononuclear cells (PBMCs) from vaccinated individuals showed more IFNγ-production in comparison to those of non-vaccinated individuals, but this did not lead to a decrease of symptoms after challenge[154].

The most recently reported T cell inducing vaccine candidates are FP-0.01 (Flunisyn™) and Multimeric-001 developed by Immune Targeting Systems Limited and BiondVax Pharmaceuticals ltd. respectively. The first vaccine consists of six peptides derived from FLUAV NP, M1, PB1 and PB2 proteins. These peptides are linked to fluorocarbon, which self-assembles into micelles omitting the need for adjuvants and increasing the peptides' *in vivo* half-life[155]. After vaccination of healthy volunteers with FP-0.01, the frequency of IFNγ-producing $CD8^+$ T cells was only slightly increased. However, PBMCs isolated from these vaccinated individuals showed an increased IFNγ-production, in comparison to unvaccinated individuals, following stimulation. Multimeric-001, is composed of a large protein comprising nine highly conserved peptides derived from FLUAV HA, NP and M1 proteins. PBMCs from vaccinated individuals show an increased proliferation and cytokine-production in comparison to PBMCs from non-vaccinated individuals after stimulation with peptides derived from influenza virus NP and HA proteins[156].

3.5 Conclusions

In order to elicit robust, long lasting, heterosubtypic immunity, there are some considerations to keep in mind when developing new vaccine candidates. Cross-reactive $CD8^+$ CTL-mediated immunity plays an important part in controlling FLUAV infections, so it would be logical to focus on inducing a potent memory $CD8^+$ T cell response. To induce such a strong $CD8^+$ T cell response, efficient antigen-presentation by APCs is important. For this, it is interesting to target relevant DC subsets—such as $CD8^+$ and $CD103^+$ DCs—for maximal FLUAV-specific CTL-stimulation[157]. The important role of DCs to activate $CD8^+$ T cells makes them attractive targets for vaccination. One approach to develop such so-called DC vaccines is by targeting antigen to PRR-activated APCs in an antibody-dependent way to achieve a strong initial T cell response[158]. Studies in animal models have demonstrated that targeting protein vaccines to lung dendritic cells even leads to the generation of Trm $CD8^+$ T cells protective against FLUAV challenge[159,160]. In humans however, little is known about the protective potential of lung Trm cells, but they are important to consider when designing a novel vaccine candidate. To generate resident memory T cells by means of vaccination, one has to keep in mind that the number of generated memory T cells is determined by the size of the burst phase of the initial T cell response. Therefore, it is essential to induce an effector T cell population that is as large as possible. Recruiting antigen-specific $CD8^+$ T cells is dependent on the presentation of antigen to the T cells, so the amount of presented antigen is crucial for the Trm cell population size[159]. In addition, the antigen-presentation at later time points is essential for generating a good Trm response. To achieve this, booster vaccines given at the correct moment might skew the Trm cell populations to higher affinity clones. A recent study showed that rapid boosting after vaccination with LAIV generated superior $CD8^+$ T cell memory[161]. Research suggests that these boosters can give rise to a robust memory T cell response as well as enhance Tfh responses, because these cells are also dependent on late antigen-encounters[162,163]. Taking the memory T cells into account, this kind of vaccination strategies will generate a better antibody response and a more robust cellular response which is cross-reactive to new influenza strains.

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The good thing about science is that it's true whether or not you believe in it.

Neil deGrasse Tyson

PART II: AIMS OF THE THESIS

For over 50 years researchers have been studying Mx proteins. Since the serendipitous discovery of the murine *Mx1* gene by Jean Lindenmann[1], a lot has evolved in this research field. Mouse *Mx1* and human *MxA* proved to be type I and III IFN-responsive genes, the products of which are involved in the antiviral immune response against an array of viruses (Reviewed by Verhelst *et al.*[2]). Especially the antiviral effects against influenza and Thogoto virus have been thoroughly investigated. In *in vitro* systems and mouse models, Mx1 and MxA reduce productive infection by these viruses[3-6]. Theoretically, every cell type which is able to induce an IFN response can express Mx1 proteins. However, until now there is no definite proof that expression of Mx1 protects immune cells against FLUAV and THOV infections, nor is there anything known about the influence of Mx1 on the subsequent adaptive immune response. The main reason for this hiatus is that most FLUAV infection studies are performed with mice which do not express a functional Mx1 protein[7]. Nevertheless, there is some indirect evidence that shows that $CD103^+$ DCs and resident memory $CD8^+$ T cells are protected against FLUAV infection due to an IFN-induced antiviral state[8,9]. By deduction, one could argue that the Mx1 protein would also have a function in this antiviral state if the used mouse strain would express a functional Mx1 protein.

To tackle this problem, we first set up a mouse model wherein we could restrict the Mx1 expression in immune cells. Since most immune cells are of hematopoietic origin, we opted to generate bone marrow chimeras by reciprocal transfer of bone marrow cells between mice with a functional or non-functional *Mx1* gene. Using this model, the first and second aim of the thesis were to investigate the effect of Mx1 in a FLUAV (first aim) and a THOV (second aim) infection model when primarily expressed by bone marrow-derived cells or stromal cells.

Altenburg and colleagues reported on the construction of recombinant modified vaccinia Ankara (rMVA)-based vaccines that drive expression of FLUAV nucleoprotein (NP) with or without modifications[10]. In that study it was apparent that mutation or removal of the nuclear localization signal (NLS) of NP or N-terminal fusion of ubiquitin to NP improved the activation of NP-specific T cell clones *in vitro*. Immunization of C57BL/6 mice with these rMVA-NP constructs elicited protection against a lethal challenge with PR8 virus, but did not reveal differences between the constructs. The differential effects *in vitro*, and the lack of these effects *in vivo*, might be explained as follows. In order to elicit a strong $CD8^+$ T cell response, the amount of processed antigen should reach a certain threshold. In C57BL/6 mice, the $CD8^+$ T cell response is dominated by $NP_{366-374}$ specific $CD8^+$ T cells, which easily reaches this threshold, irrespective of the modification of NP. It is known that NP is targeted by mouse Mx1 and even interacts with Mx1[11]. By this, Mx1 could be a factor that limits the amount of processed antigen. Therefore, as a third aim of the thesis, we wanted to compare

the immunogenicity of these rMVA-NP vaccines in a mouse strain which does or does not express a functional Mx1 protein (B6.A2G $Mx1^{+/+}$ or B6.A2G $Mx1^{-/-}$).

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Carry on my wayward son
For there'll be peace when you are done
Lay your weary head to rest
Don't you cry no more

Kansas

PART III: RESULTS

Wat je vandaag moet doen, moet je doen zoals je morgen denkt dat je het had moeten doen.
Toon Hermans

CHAPTER 4: Mx1 expression in bone marrow-derived cells has an important function in the resistance against Thogoto virus infection, but not against influenza A virus infection

Mx1 expression in bone marrow-derived cells has an important function in the resistance against Thogoto virus infection, but not against influenza A virus infection

Running title: Elucidating the role of Mx1 in the immune cell compartment

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Relative contributions of the authors:

JS, LVH and KR performed the experiments. JS, LVH, KR and XS designed the experiments. KR and XS supervised the research. JS, KR and XS co-wrote the manuscript.

Abstract

The antiviral myxovirus resistance 1 (*Mx1*) gene is an interferon-induced gene which encodes a GTPase that plays an important role in the defense of mammalian cells against influenza A and other viruses. *Mx1* provides protection against a number of viral infections, and this protection is independent of the expression of other interferon-induced genes. Because of their rapid induction by interferon and their antiviral function, *Mx* genes are considered an important part of the innate immune response. The way in which the immune cell compartment against influenza A virus is influenced or managed by Mx proteins, however, has not been investigated in detail. To determine if the murine Mx1 protein has a role in the immune cell compartment, we performed bone marrow chimera experiments using congenic B6.A2G $Mx1^{+/+}$ and B6.A2G $Mx1^{-/-}$ mice, which do or do not express a functional *Mx1* gene respectively. Bone marrow chimeric mice were challenged with influenza A virus PR8, and the effect of the allogeneic immune compartment within the $Mx1^{+/+}$ or $Mx1^{-/-}$ background was assessed using different viral and immunological readouts. This model showed that the role of Mx1 protein in the stromal cells prevails in the protection against influenza A virus. The same chimeric model was used in conjunction with Thogoto virus challenge. In this model it was apparent that Mx1 proteins can play a role of significance in the immune compartment.

4.1 Introduction

Worldwide, influenza virus causes three to five million severe cases of illness and an estimate of 250 000 to 500 000 fatal cases every year[1]. When people get infected with influenza virus, the first cells that are targeted, are the airway epithelial cells. After binding, endocytosis and membrane fusion, the viral ribonucleoproteins (vRNPs) are released into the cytoplasm. These then enter the nucleus, where transcription and replication will take place (reviewed by Engelhardt *et al.*[2] and Lakadamyali *et al.*[3]). These incoming vRNPs first form viral mRNA (primary transcription), which is then transported to the cytosol for translation into proteins. The newly produced viral proteins go back to the nucleus to start the replication of the viral genome. The resulting progeny viral RNA molecules form vRNPs, and leave the nucleus, ready for packaging and budding[2].

Belonging to the same family of *Orthomyxoviridae,* Thogoto virus (THOV) is a tick-borne virus first isolated in the Thogoto forest near Nairobi, Kenia[4]. THOV is genetically related to influenza viruses, but in contrast to influenza virus, small rodents are a natural host of this virus[5]. Antibodies against THOV have been detected in rats, buffaloes, camels, donkeys, cattle, sheep and humans[6]. However, infections in humans are rare[7].

Myxovirus resistance 1 (Mx1) proteins are interferon-induced dynamin-like large GTPases that can inhibit a wide array of viruses such as *Orthomyxoviridae*, *Rhabdoviridae* and *Bunyaviridae*. Especially the antiviral effect on influenza A virus (FLUAV) and THOV has been studied elaborately (reviewed by Verhelst *et al.*[8]). The exact mechanism by which Mx1 proteins inhibit viral infection is however still largely unknown. Murine Mx1 inhibits influenza A viruses by suppressing primary transcription of viral genes in the nucleus[9]. Previously, we showed that murine Mx1 interacts with the polymerase basic 2 (PB2) protein and nucleoprotein (NP) in vRNPs, and disturbs their interaction[10]. Based on this study, and on the observation that human MxA proteins – like dynamins – form ring-like structures[11-15], we hypothesized that the interaction with PB2 and NP might be mediated by an Mx1 oligomeric ring, which actively disrupts the PB2-NP interaction[10]. In addition, we recently demonstrated that Mx1 can not only prevent formation of vRNPs, but it can also disrupt existing vRNPs, which is in line with the proposed model[16].

Although there is a body of evidence that Mx proteins give protection in the innate immune response against certain viral infection, little is known about the possible role of Mx proteins in the different immune cell types. Previous studies which could not define a clear role for Mx1 in the immune cell compartment discussed only one or a few different immune cell types, or experiments were performed using mouse strains which do not express a

functional Mx1 protein[17-20]. In contrast, several research groups reported about the role of the type I IFN response in immune cells after FLUAV infection, leading to the formation of an antiviral state in different immune cell types, rendering them resistant against influenza virus infection[21,22], or increasing the susceptibility of certain immune cells by an attenuated IFNAR-response[23]. Taken together, these studies raise a lot of extra questions about the role of the type I IFN response, and in particular the role of the Mx1 protein, in immune cells following a FLUAV infection. Therefore, we investigated the role of *Mx1* expression in immune cells in an *in vivo* infection model using radiation chimeric mice. We show that the response to influenza virus infection is largely independent of the genotype of the hematopoietic cells, while the genotype of the stromal cells seems to determine the better part of the Mx1-driven protection against influenza virus. Conversely, this is not the case when using a Thogoto virus infection model, where we observed a role for both the genotype of the recipient and the donor.

4.2 Results

4.2.1 B6.A2G Mx1$^{+/+}$ mice are resistant against infection with a high dose of influenza A virus

Homozygous *Mx1* positive (Mx1$^{+/+}$) and *Mx1* negative (Mx1$^{-/-}$) B6.A2G mice were infected intranasally with 20 times the median lethal dose (LD_{50}) of a mouse-adapted influenza A/Puerto Rico/8/34 (maPR8) lab strain. Morbidity and mortality were monitored during 14 days post infection (dpi). The effect of virus infection is clearly seen for the Mx1$^{-/-}$ group, where severe weight loss is observed and all mice succumbed to infection by day 9 post infection (Figure 4.1). In contrast, all Mx1$^{+/+}$ mice survived infection, and did not show significant weight loss. This illustrates that Mx1$^{+/+}$ B6.A2G mice are highly resistant to influenza infections, even at high doses.

4.2.2 Generation of bone marrow chimeras

Most immune cells have a hematopoietic origin. Replacing bone marrow cells from mice that express a functional Mx1 protein (B6.A2G Mx1$^{+/+}$) with those from mice which do not express a functional form of Mx1 (B6.A2G Mx1$^{-/-}$) allows us to determine the possible role for the Mx1 protein in the immune cell compartment after a viral infection. For this, B6.A2G mice (Mx1$^{+/+}$ or Mx1$^{-/-}$) were lethally irradiated (10 Grey), and were reconstituted 24h later with syngeneic or allogeneic bone marrow cells. Experimental transfers were as follows: B6.A2G Mx1$^{-/-}$ donors into B6.A2G Mx1$^{-/-}$ recipients (Mx1$^{-/-}$ → Mx1$^{-/-}$), B6.A2G Mx1$^{-/-}$ donors into B6.A2G Mx1$^{+/+}$ recipients (Mx1$^{-/-}$ → Mx1$^{+/+}$), B6.A2G Mx1$^{+/+}$ donors into B6.A2G Mx1$^{+/+}$ recipients (Mx1$^{+/+}$ → Mx1$^{+/+}$), and B6.A2G Mx1$^{+/+}$ donors into B6.A2G Mx1$^{-/-}$ recipients (Mx1$^{+/+}$ → Mx1$^{-/-}$). A schematic overview of the different experimental groups is shown in Figure 4.2.

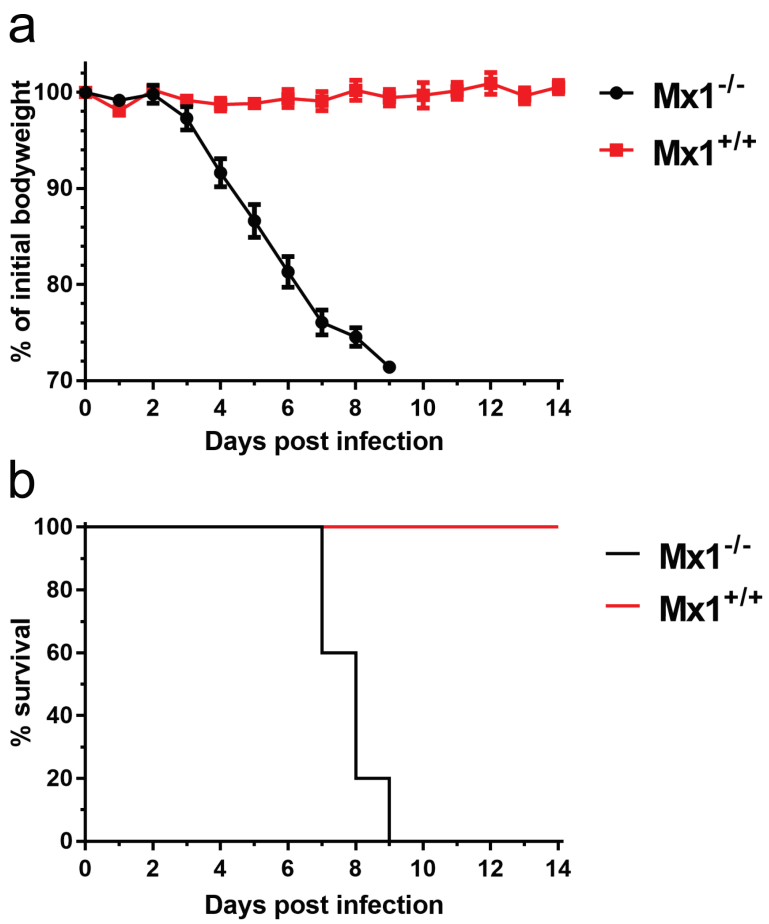

Figure 4.1 Morbidity and mortality after FLUAV infection of Mx1$^{-/-}$ and Mx1$^{+/+}$ B6.A2G mice. Mice were infected intranasally with 20 LD_{50} of maPR8. Morbidity **(a)** and mortality **(b)** were monitored during 14 days post infection. Data points were obtained in one single experiment and represent the average of five mice. Error bars represent the standard error of the mean.

4.2.3 Resistance to influenza A virus infection primarily depends on the genotype of the recipient

Bone marrow chimeric mice were infected with 10 LD_{50} of maPR8, and bodyweight and morbidity were monitored during six days after infection. B6.A2G Mx1$^{-/-}$ recipient mice (Mx1$^{-/-}$ → Mx1$^{-/-}$ and Mx1$^{+/+}$ → Mx1$^{-/-}$) clearly suffer more from the consequences of the infection than B6.A2G Mx1$^{+/+}$ recipient mice (Mx1$^{+/+}$ → Mx1$^{+/+}$ and Mx1$^{-/-}$ → Mx1$^{+/+}$), losing significantly more bodyweight after infection (Figure 4.3a). At 3 dpi, half of the mice of each group was sacrificed, and lung viral titers were determined. The same was done at 6 dpi for the second half of each group. Viral titers in the lung were about tenfold lower in B6.A2G Mx1$^{+/+}$ recipients than in B6.A2G Mx1$^{-/-}$ recipients on day three after infection (Figure 4.3b). Three days later, at 6 dpi, a trend of lower lung viral titers was observed in the B6.A2G Mx1$^{+/+}$ recipient groups in comparison to the B6.A2G Mx1$^{-/-}$ recipients. Over the course of

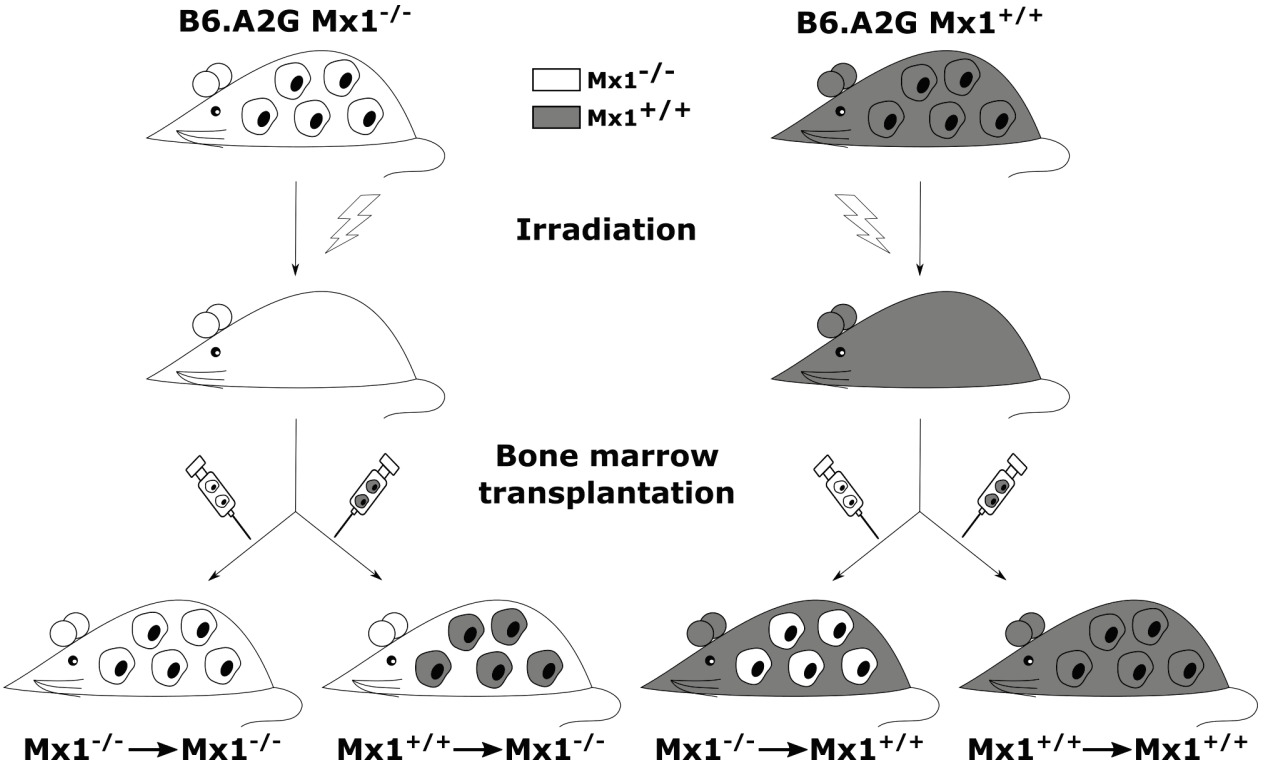

Figure 4.2 Schematic overview of the generation of the different bone marrow chimeric groups

time, the B6.A2G Mx1$^{+/+}$ recipients differ significantly from the B6.A2G Mx1$^{-/-}$ recipients both in morbidity and lung viral titers.

Since Mx1 has been shown to inhibit primary transcription of viral genes in the nucleus[9], we determined viral mRNA levels in the lung at three different time points by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). For this, several mice of each challenged bone marrow chimeric group were sacrificed either prior to infection (0 dpi), or at 3, or at 6 dpi. Lungs were isolated and homogenized, and viral mRNA was extracted to determine the levels by RT-qPCR. Three days after infection, viral mRNA levels were much lower in lungs of B6.A2G Mx1$^{+/+}$ recipients compared to those in lungs of B6.A2G Mx1$^{-/-}$ recipient mice (Figure 4.4a). At 6 dpi, this difference was even more profound. Pavlovic *et al.* showed that Mx1 differentially inhibits the transcription of the different influenza virus segments. The strongest inhibition is observed for the largest transcripts (PB1, PB2, and PA), but the smallest transcripts (M and NS) are barely inhibited[9]. In contrast, we noticed that the inhibiting effect of Mx1 was equally strong for the shorter and the longer viral segments. This effect was also seen for viral protein levels in the lung, as determined by western blot analysis of total lung homogenates (Figure 4.4b). The virus NP and M2 protein levels were both strongly affected by Mx1 activity in B6.A2G Mx1$^{+/+}$ recipient mice. Although Mx1 was well expressed in the lungs of the Mx1$^{+/+}$ → Mx1$^{-/-}$

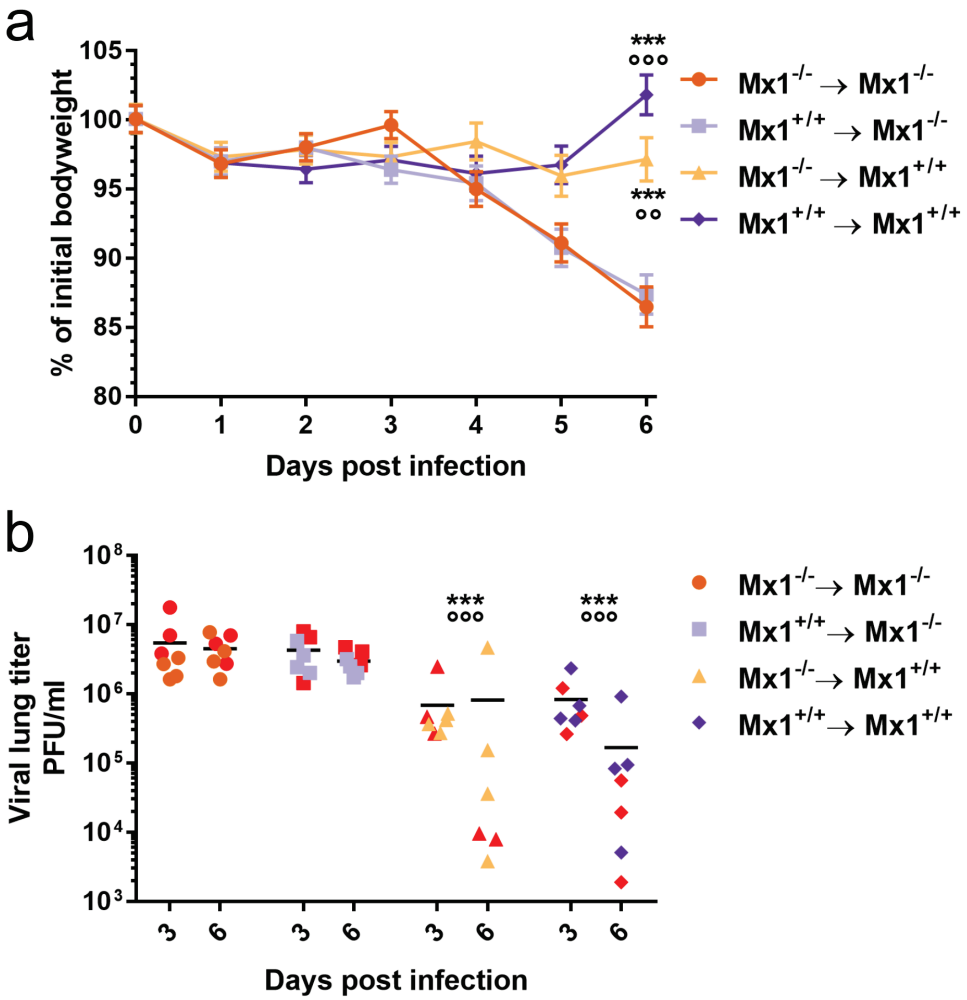

Figure 4.3 Morbidity and viral lung titers of bone marrow chimeric mice after FLUAV infection. Mice were infected with 10 LD_{50} of maPR8. **(a)** Body weight was monitored during 6 days after infection. Data points represent the average of at least six mice. Error bars represent the standard error of the mean. Asterisks indicate the significant difference with the $Mx1^{-/-}$ → $Mx1^{-/-}$ group over time. ***, $p < 0.001$. Circles indicate the significant difference with the $Mx1^{+/+}$ → $Mx1^{-/-}$ group over time. °°, $p < 0.01$; °°°, $p < 0.001$. **(b)** Half of the infected mice of each group were sacrificed at 3 dpi, and the other half at 6 dpi. At both time points lung viral titers were determined. Each data point represents the lung viral titer of a single animal. Red data points represent samples depicted in Figure 4.4b. Asterisks indicate the significant difference with the $Mx1^{-/-}$ → $Mx1^{-/-}$ group. ***, $p < 0.001$. Circles indicate the significant difference with the $Mx1^{+/+}$ → $Mx1^{-/-}$ group. °°°, $p < 0.001$. Data are pooled from 2 independently performed experiments.

group, there was no clear effect on viral infection. IFITM3, which is an IFN-induced protein, is already expressed well at steady state conditions. This high baseline expression makes it difficult to distinguish differences in expression levels between the four bone marrow chimera groups upon FLUAV infection. Nevertheless, it conveys the impression that IFITM3 expression is lower in $Mx1^{+/+}$ recipients, especially in the $Mx1^{+/+}$ → $Mx1^{+/+}$ group. These data suggest that Mx1 expression by stromal cells has a stronger effect on FLUAV infection than Mx1 expression by cells with a hematopoietic origin.

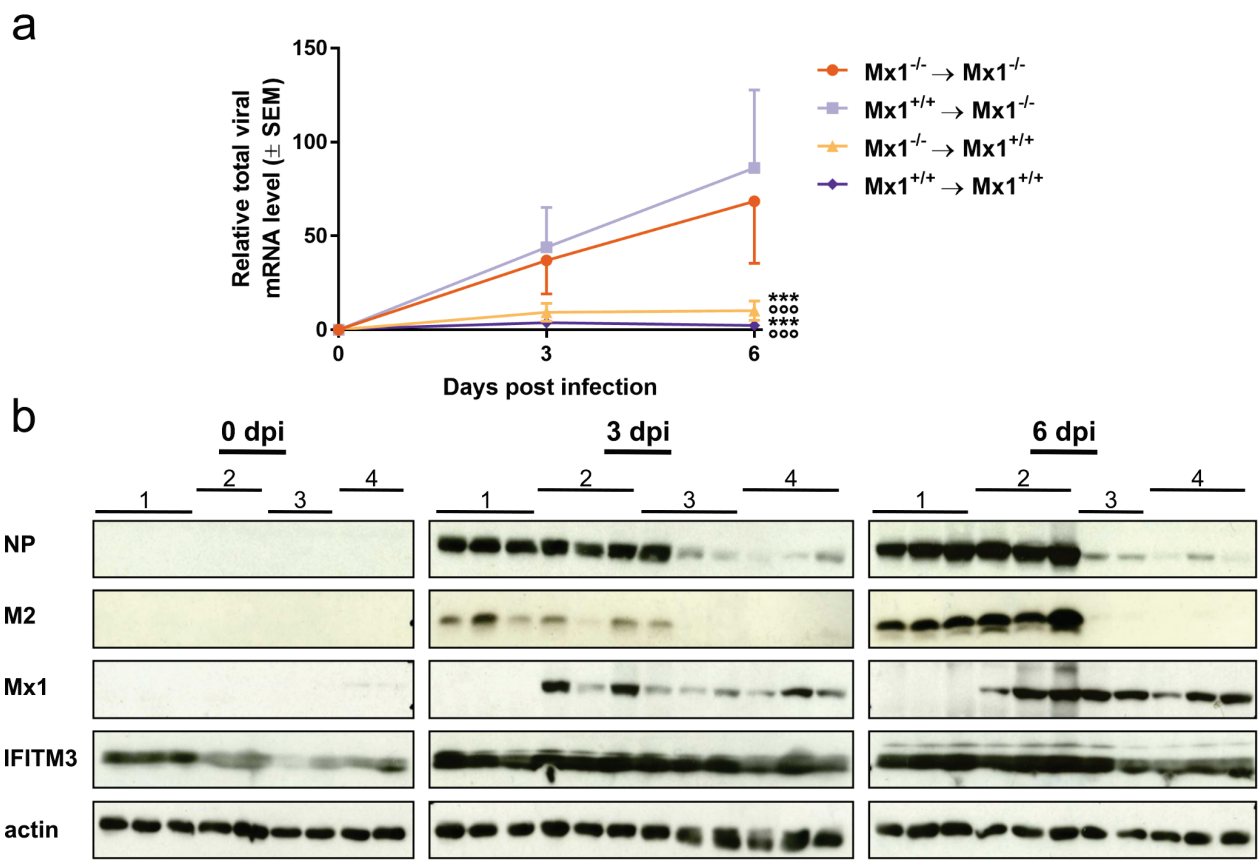

Figure 4.4 Viral mRNA and protein levels of bone marrow chimeric mice after FLUAV infection. Mice were infected with 10 LD_{50} of maPR8. Part of each group was sacrificed at 0, 3 or 6 dpi, lungs were isolated and processed to lung homogenates. **(a)** From these homogenates total mRNA was isolated, and this mRNA was used to determine viral mRNA levels by RT-qPCR. Total viral mRNA was quantified for each lung homogenate by RT-qPCR. Data points represent average levels of total viral mRNA relative to household genes. Error bars represent standard error of the mean. Asterisks indicate the significant difference with the $Mx1^{-/-}$ → $Mx1^{-/-}$ group. ***, $p < 0.001$. Circles indicate the significant difference with the $Mx1^{+/+}$ → $Mx1^{-/-}$ group. °°°, $p < 0.001$. Data are pooled from 2 independently performed experiments. **(b)** Lung homogenates were used for western blot analysis of viral proteins NP and M2, and interferon-induced cellular proteins Mx1 and IFITM3. Actin was detected as loading control. Samples from different bone marrow chimera groups are numbered from 1 to 4. **1**, $Mx1^{-/-}$ → $Mx1^{-/-}$; **2**, $Mx1^{+/+}$ → $Mx1^{-/-}$; **3**, $Mx1^{-/-}$ → $Mx1^{+/+}$; **4**, $Mx1^{+/+}$ → $Mx1^{+/+}$. Data are representative of two similar experiments.

4.2.4 Cellular responses after influenza A virus infection differ between $Mx1^{-/-}$ and $Mx1^{+/+}$ recipient mice

Expression of a functional Mx1 protein in bone marrow-derived cells does not seem to contribute significantly to the resistance against FLUAV infection. Mx1 expression in the stromal cells, on the other hand, appears imperative for this resistance. However, nothing is known about the impact of Mx1 expression on the formation and shape of the immune response following FLUAV infection. To study this, lungs of FLUAV-infected bone marrow chimeric mice were isolated at three different time points (0, 3 and 6 dpi), single cell

suspensions were prepared and stained for different surface markers. This allowed us to distinguish various immune cell types by flow cytometry (Supplementary figure S4.1). Prior to infection (0 dpi), the levels of all analyzed immune cell types were not significantly different between the four chimeric groups (Supplementary figures S4.2 and S4.3). This suggests that the composition of the immune compartment is comparable at steady state conditions irrespective of the genotype of the immune and stromal cells.

During the course of infection, neutrophil levels behave quite similar in all bone marrow chimeric groups (Figure S4.2a and S4.3a). The $Mx1^{-/-}$ → $Mx1^{-/-}$ group shows the highest neutrophil influx at 3 and 6 dpi. Over time, the neutrophil level of the $Mx1^{-/-}$ → $Mx1^{-/-}$ group differs significantly with that of the $Mx1^{-/-}$ → $Mx1^{+/+}$ group, which shows the lowest level of all at 6 dpi. In contrast to neutrophils, eosinophils get depleted over time (Figure S4.2b and S4.3b). This depletion over time is stronger for $Mx1^{-/-}$ recipients than it is for $Mx1^{+/+}$ recipients, and is most clearly seen at 6 dpi. The lung conventional dendritic cell (cDC) levels are very low, which makes it difficult to draw straightforward conclusions. The level of cDC1s in $Mx1^{-/-}$ → $Mx1^{-/-}$ lungs differs significantly over time from the three other groups, probably because of the initial higher cDC1 levels at 0 dpi in this group (Figure S4.2c and S4.3c). However, it is noteworthy to mention that there is quite a big spread in the data of this group at this time point (Figure S4.3). The four bone marrow chimeric groups show no significant differences over time for their cDC2 levels (Figure S4.2d and S4.3d).

After FLUAV infection, dendritic cells take up viral particles or virus infected cells, and migrate to the draining lymph nodes. There, they present this antigen and trigger an antigen-specific T cell response[24]. T cell levels were also measured at 0, 3 and 6 dpi, and compared between the bone marrow chimeric groups. The $CD4^+$ T cell levels in the lungs of the $Mx1^{-/-}$ → $Mx1^{-/-}$ group differ significantly over time from all other groups (Figure S4.2e and S4.3e). This is because the $CD4^+$ T cell levels in this group drop over time, while in the other groups the levels remained approximately the same. In contrast, the $CD8^+$ T cell levels increase similarly over time for all chimeric groups (Figure S4.2f and S4.3f).

During the course of infection the clearest divergence seen between the B6.A2G $Mx1^{-/-}$ and B6.A2G $Mx1^{+/+}$ recipient mice was in the monocyte-derived dendritic cell (moDC) population (Figure 4.5a and 4.5c). We observed higher levels of moDCs in the lungs of B6.A2G $Mx1^{-/-}$ recipients in comparison to the lungs of B6.A2G $Mx1^{+/+}$ recipient mice. Previously it has been shown that, upon FLUAV infection, monocytes are massively recruited into the lung to the site of inflammation, where they differentiate into moDCs[25]. Alveolar macrophages (AMs), on the other hand, get depleted after influenza A virus

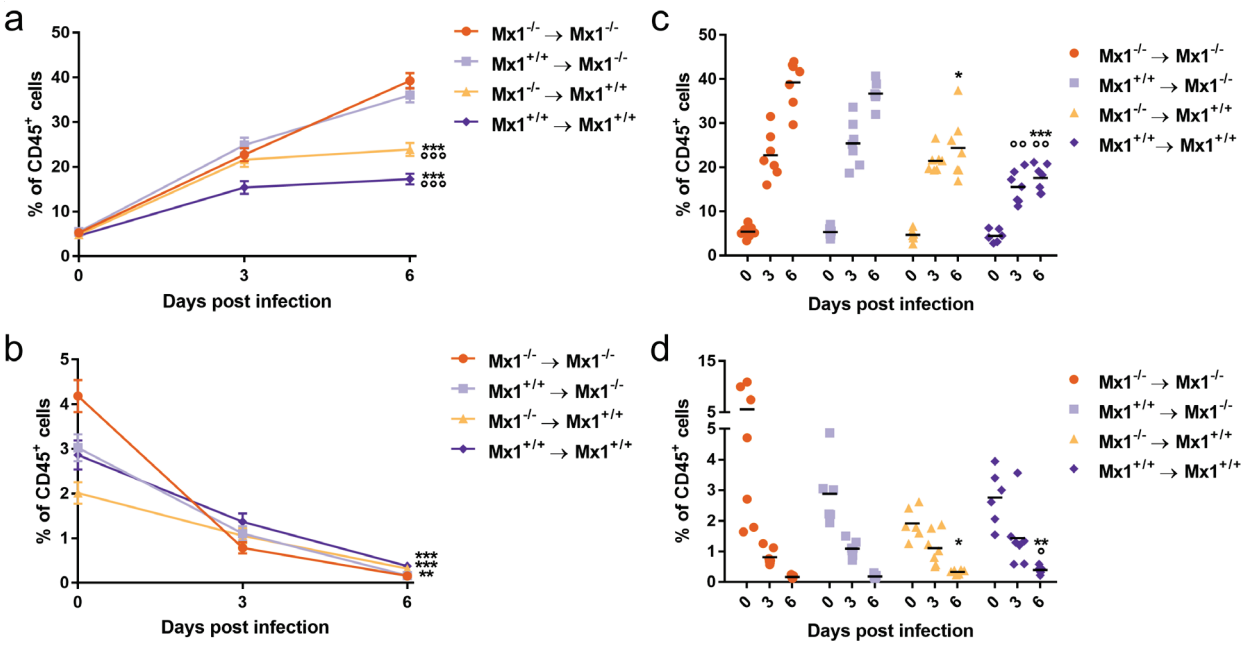

Figure 4.5 Effect of FLUAV infection on moDC and AM levels in the lung. Bone marrow chimeric mice were infected with 10 LD_{50} of maPR8, and lungs were isolated at 0, 3 and 6 dpi. These lungs were processed to single cell suspensions, which were stained for different surface markers. This allows us to identify different cell types such as monocyte-derived dendritic cells **(a and c)** and alveolar macrophages **(b and d)**. **(a and b)** Data points represent the average level of at least six mice. Error bars represent standard error of the mean. **(c and d)** Each data point represents the relative amount of cells of each indicated cell type of a single animal. Asterisks indicate the significant difference with the $Mx1^{-/-}$ → $Mx1^{-/-}$ bone marrow chimera group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Circles indicate the significant difference with the $Mx1^{+/+}$ → $Mx1^{-/-}$ bone marrow chimera group over time. °, $p < 0.05$; °°, $p < 0.01$; °°°, $p < 0.001$. Data are pooled from 2 independently performed experiments.

infection[25,26]. This was also observed in the lungs of the four different bone marrow chimera types (Figure 4.5b and 4.5d). However, the depletion of AMs over time is less strong for B6.A2G $Mx1^{+/+}$ recipients compared to B6.A2G $Mx1^{-/-}$ recipient mice. This suggests that inflammation in the lungs of $Mx1^{-/-}$ recipients is more fierce than in the $Mx1^{+/+}$ lungs after FLUAV infection.

4.2.5 Mx1 expression in bone marrow-derived cells does not influence the formation of an antigen-specific $CD8^+$ T cell response after an influenza A virus infection

Since Mx1 expression in stromal cells alleviates the disease burden in mice (Figure 4.3a), it is tempting to argue that Mx1's innate function, being the early containment of the virus, is its main role in the anti-FLUAV immune response. If the virus is inhibited early in the

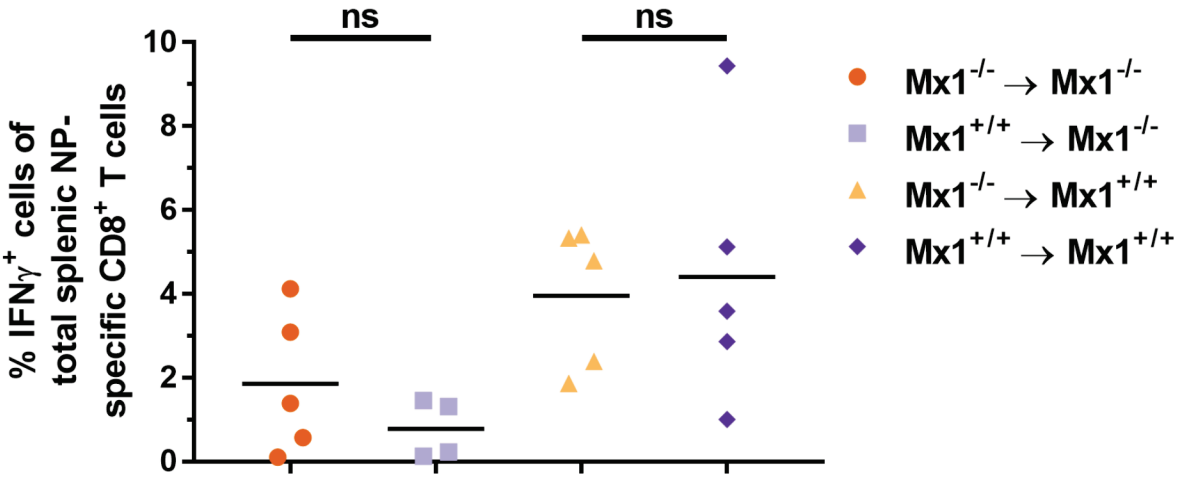

Figure 4.6 Antigen-specific CD8⁺ T cell response after FLUAV infection of bone marrow chimeric mice. B6.A2G Mx1$^{-/-}$ and B6.A2G Mx1$^{+/+}$ recipient mice were infected with either 0.5 LD_{50} and 10 LD_{50} of maPR8, respectively. Ten days after challenge, splenocytes were prepared and restimulated *ex vivo* for 16 h with a FLUAV NP-derived peptide. After restimulation, flow cytometric analysis of IFNγ-producing CD8⁺ T cells was performed. Data are obtained in one single experiment. Statistical analysis was performed using a two-sided Mann-Whitney U test. ns = not significant.

course of infection, this would suggest that there is no formation of a strong cellular antiviral response, since elucidating a specific T cell response requires a certain amount of productive infection[27]. We examined the total CD4⁺ and CD8⁺ T cell populations of bone marrow chimeras at different time points (0, 3 and 6 dpi) after maPR8 infection, but we did not observe a strong influx of T cells in any of the chimera groups (Supplementary Figures S4.2 and S4.3). Likely, the chosen time points (0, 3 and 6 dpi) are not well suited for detection of a T cell response because they are situated too early after infection. To address this issue, the specific antiviral T cell response was examined in bone marrow chimeras ten days after infection with maPR8. Because the sensitivity to FLUAV infection differs strongly between B6.A2G Mx1$^{-/-}$ recipient (Mx1$^{-/-}$ → Mx1$^{-/-}$, Mx1$^{+/+}$ → Mx1$^{-/-}$) and B6.A2G Mx1$^{+/+}$ recipient mice (Mx1$^{+/+}$ → Mx1$^{+/+}$, Mx1$^{-/-}$ → Mx1$^{+/+}$), we adjusted the dose of virus to a sublethal level for each recipient genotype (0.5 LD_{50} for Mx1$^{-/-}$ recipients and 10 LD_{50} for Mx1$^{+/+}$ recipients). Antigen-specific CD8⁺ T cell responses were analyzed in splenocytes, isolated ten days after infection. Both Mx1$^{-/-}$ and Mx1$^{+/+}$ recipients showed an NP-specific T cell response (Figure 4.6). NP-specific CD8⁺ T cell levels were higher in the B6.A2G Mx1$^{+/+}$ recipient mice compared to the B6.A2G Mx1$^{-/-}$ recipient mice (Figure 4.6), which is not surprising when considering the higher virus dose given to Mx1$^{+/+}$ recipient mice. This indicates that B6.A2G Mx1$^{+/+}$ recipients can mount a specific antiviral T cell response after FLUAV infection, and that Mx1 protein expression in stromal cells does not completely inhibit FLUAV infection nor the subsequent cellular immune response. Notably, the NP-specific CD8⁺ T cell responses in the two Mx1$^{-/-}$ recipient groups were not significantly

different from one another (Figure 4.6). The same was true for the two Mx1$^{+/+}$ recipient groups. This suggests that expression of a functional Mx1 protein in cells with a hematopoietic origin does not influence, negatively or positively, the formation of a potent cellular antiviral response.

4.2.6 B6.A2G Mx1$^{+/+}$ bone marrow reduces THOV-associated effects in B6.A2G Mx1$^{-/-}$ recipient mice

Based on the previous results, we observed no clear-cut effect of Mx1 expression in immune cells after FLUAV infection. Instead, it was apparent that expression of Mx1 in the stromal

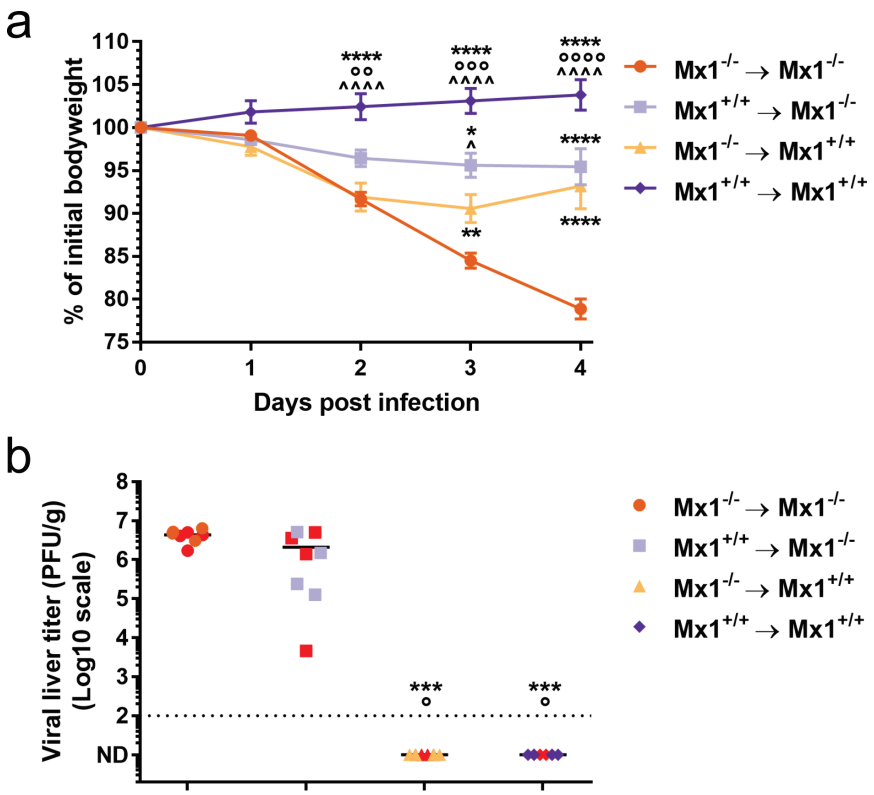

Figure 4.7 Morbidity and viral lung titers of bone marrow chimeric mice after THOV infection. Mice were infected intraperitoneally with 1000 PFU of THOV. **(a)** Body weights were monitored during 4 days after infection. Data points represent the average of eight mice. Error bars represent the standard error of the mean. Statistical analysis was done using a two-way ANOVA with *post hoc* Tukey's HSD test. Asterisks indicate the significant difference with the Mx1$^{-/-}$ → Mx1$^{-/-}$ group. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$. Circles indicate the significant difference with the Mx1$^{+/+}$ → Mx1$^{-/-}$ group. °°, $p < 0.01$; °°°, $p < 0.001$; °°°°, $p < 0.0001$. Caps indicate the significant difference with the Mx1$^{-/-}$ → Mx1$^{+/+}$ group. ^, $p < 0.05$; ^^^^, $p < 0.0001$. **(b)** Four days post infection, liver viral titers were determined. Each data point represents the liver viral titer of a single animal. Red data points represent samples depicted in Figure 4.8. ND = not detectable. Statistical analysis was performed using Kruskal-Wallis test with *post hoc* Dunn's multiple comparison test. Asterisks indicate the significant difference with the Mx1$^{-/-}$ → Mx1$^{-/-}$ group. ***, $p < 0.001$. Circles indicate the significant difference with the Mx1$^{+/+}$ → Mx1$^{-/-}$ group. °, $p < 0.05$. Data are pooled from 2 independently performed experiments.

cells is the main driving force for resistance against FLUAV infection. A possible explanation could have been that the direct innate effect of Mx1 is too strong to allow the development of an adequate cellular immune response in mice expressing Mx1 in their stromal cells. However, this possibility was debunked by the observation of a strong antigen-specific $CD8^+$ T cell response in the B6.A2G $Mx1^{+/+}$ recipient mice after FLUAV infection (Figure 4.6). Another possibility might be that the influenza virus infection model is not well suited for eliciting the role of Mx1 expression in immune cells. In this regard, another *in vivo* infection model was set up using the Thogoto virus (THOV), an Orthomyxovirus that is also sensitive to Mx1.

Bone marrow chimeric mice were infected intraperitoneally with 1000 plaque forming units (PFU) of THOV (Sicilian SiAr 126 isolate), and body weight was monitored once daily for four days after infection. The effect of THOV infection on $Mx1^{-/-} \rightarrow Mx1^{-/-}$ and $Mx1^{+/+} \rightarrow Mx1^{+/+}$ mice was as expected in the sense that $Mx1^{+/+} \rightarrow Mx1^{+/+}$ mice showed no body weight loss, and $Mx1^{-/-} \rightarrow Mx1^{-/-}$ mice lost quite some body weight and became moribund by day 4 post infection (Figure 4.7a). Interestingly, the $Mx1^{-/-} \rightarrow Mx1^{+/+}$ and $Mx1^{+/+} \rightarrow Mx1^{-/-}$ groups responded differently than in the influenza virus infection model. B6.A2G $Mx1^{+/+}$ mice that had received $Mx1^{-/-}$ bone marrow ($Mx1^{-/-} \rightarrow Mx1^{+/+}$) lost significantly more body weight than B6.A2G $Mx1^{-/-}$ mice that had received $Mx1^{+/+}$ bone marrow ($Mx1^{+/+} \rightarrow Mx1^{-/-}$), indicating a protective role for Mx1 expression in hematopoietic cells in this infection model. Surprisingly, liver viral titers do not reflect these findings. Just like the lung viral titers in the FLUAV infection model, the liver viral titers of both B6.A2G $Mx1^{-/-}$ recipient groups are very high (approximately 10^6 to 10^7 PFU/g), and they are significantly lower (below the detection limit of 10^2 PFU/g) for B6.A2G $Mx1^{+/+}$ recipient groups at 4 dpi (Figure 4.7b). Notably however, $Mx1^{+/+} \rightarrow Mx1^{-/-}$ mice showed a twofold lower average viral titer than the $Mx1^{-/-} \rightarrow Mx1^{-/-}$ group. These data suggest that Mx1 expression primarily in hematopoietic cells can significantly reduce the THOV-associated body weight loss in B6.A2G $Mx1^{-/-}$ recipient mice.

Four days after infection, Mx1 protein expression in the liver was determined by western blot analysis of total liver homogenates. As expected, no Mx1 expression was observed in the $Mx1^{-/-} \rightarrow Mx1^{-/-}$ livers (Figure 4.8). In contrast, $Mx1^{+/+} \rightarrow Mx1^{-/-}$ mice do express Mx1 in their liver, however, the expression levels of the $Mx1^{-/-} \rightarrow Mx1^{+/+}$ mice seem much higher. This is not surprising, considering that $Mx1^{+/+} \rightarrow Mx1^{-/-}$ mice only express Mx1 in cells from hematopoietic origin, and $Mx1^{-/-} \rightarrow Mx1^{+/+}$ mice can express Mx1 in all their cells except those from hematopoietic origin. Given this, it is quite remarkable that mice from the $Mx1^{+/+} \rightarrow Mx1^{+/+}$ group, which can express functional Mx1 protein in all of their cell types, do not express high levels of Mx1 after THOV infection. The reason for this is the absence of

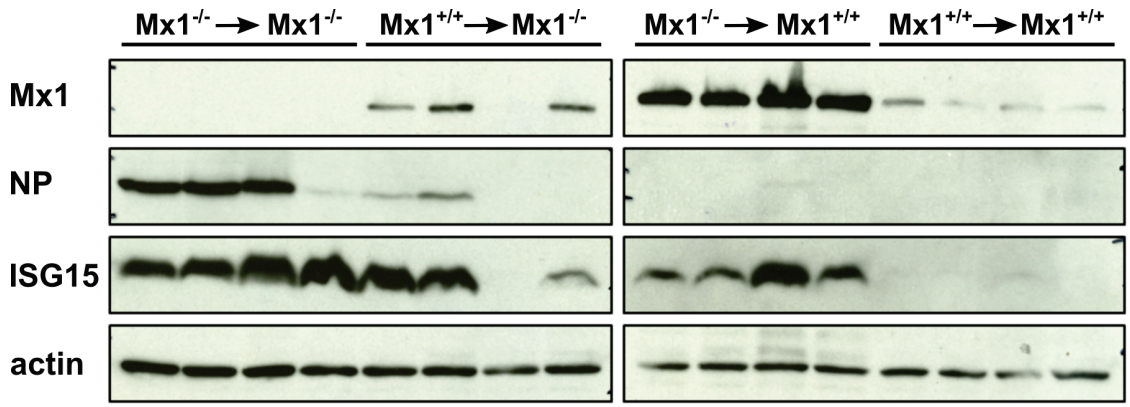

Figure 4.8 Mx1 protein levels in livers of bone marrow chimeric mice after THOV infection. Bone marrow chimeric mice were infected intraperitoneally with 1000 PFU of THOV. Four days after infection livers were isolated and processed to liver homogenates. These liver homogenates were used for western blot analysis of Mx1, THOV NP, and ISG15. Actin was detected as loading control. Data are representative of two similar experiments.

a strong interferon response in the liver, as illustrated by the undetectable ISG15 expression in the liver homogenates from this chimeric group. In the $Mx1^{-/-}$ → $Mx1^{+/+}$ group, as well as in both $Mx1^{-/-}$ recipient groups, we observed ISG15 expression indicating an adequate interferon response. This interferon response is presumably induced by the THOV infection. However, we only observe strong expression of THOV NP in the $Mx1^{-/-}$ → $Mx1^{-/-}$ liver homogenates. $Mx1^{+/+}$ → $Mx1^{-/-}$ homogenates show very little to no expression of the THOV NP even though we measured high liver viral titers in this group. It is not surprising that we do not see any THOV NP expression in the livers of $Mx1^{+/+}$ recipient mice since they also show no virus growth in the liver.

4.2.7 B6.A2G $Mx1^{+/+}$ bone marrow-derived cells can ameliorate THOV-induced liver pathology in B6.A2G $Mx1^{-/-}$ recipients

THOV infection was previously shown to cause severe liver pathology in $Mx1^{-/-}$ mice, while $Mx1^{+/+}$ mice did not show any liver pathology[28]. In order to examine the effect of THOV on liver tissue of bone marrow chimeric mice, livers of these mice were isolated four days after THOV infection and prepared for histological analysis. Macroscopically, the livers of the $Mx1^{-/-}$ → $Mx1^{-/-}$ mice appeared very different from the livers of the other chimera groups (Figure 4.9a). The $Mx1^{-/-}$ → $Mx1^{-/-}$ livers were very pale and friable in comparison to the other livers which had a normal brown-red color and firm tissue. Tissue sections were stained with hematoxylin and eosin (H&E). Microscopically, H&E-stained $Mx1^{-/-}$ → $Mx1^{-/-}$ liver tissue showed lesions characterized by focal to widespread liver cell necrosis (Figure 4.9b). Interestingly, $Mx1^{+/+}$ → $Mx1^{-/-}$ liver tissue also showed lesions, however, these lesions appear to be in an earlier stage of necrosis than the lesions in $Mx1^{-/-}$ → $Mx1^{-/-}$ liver tissue. This is manifested as foci in the liver tissue where structure and cell architecture are lost, and strong immune cell influx was observed. This suggests that Mx1 expression in cells with hematopoietic origin can reduce or at least delay liver pathology in B6.A2G $Mx1^{-/-}$

recipient mice. B6.A2G Mx1$^{+/+}$ recipient's liver tissue was normal and showed no lesions (Figure 4.9b).

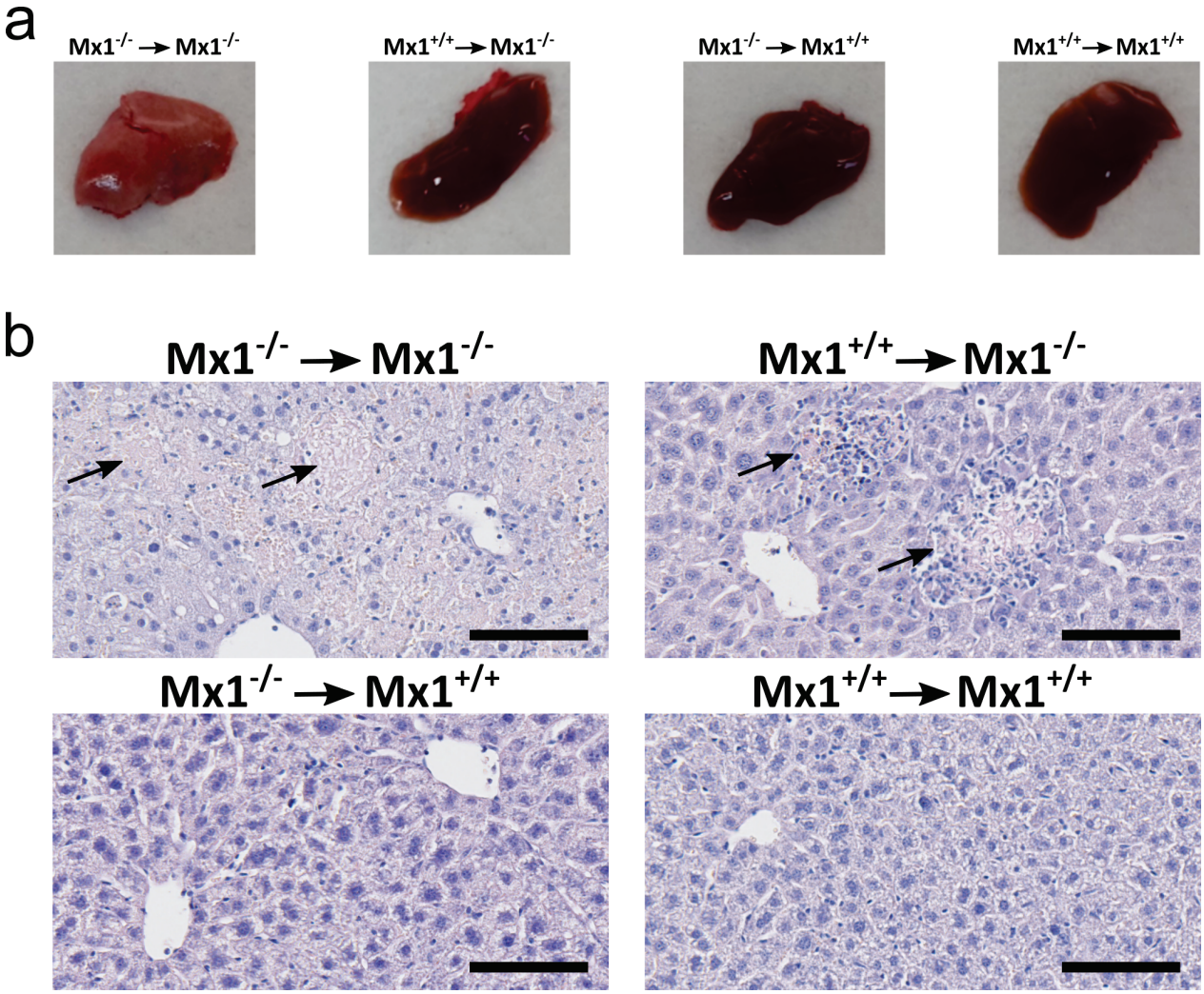

Figure 4.9 Macro- and microscopic view of liver tissue of bone marrow chimeric mice after THOV infection. Bone marrow chimeric mice were infected intraperitoneally with 1000 PFU of THOV. Four days after infection livers were isolated, photographed, and prepared for histological analysis. **(a)** Macroscopic view of livers. **(b)** Microscopic view of liver tissue stained with hematoxylin and eosin. Arrows indicate focal zones of liver cell necrosis. Scale bar = 100 µm. Data are representative of two similar experiments.

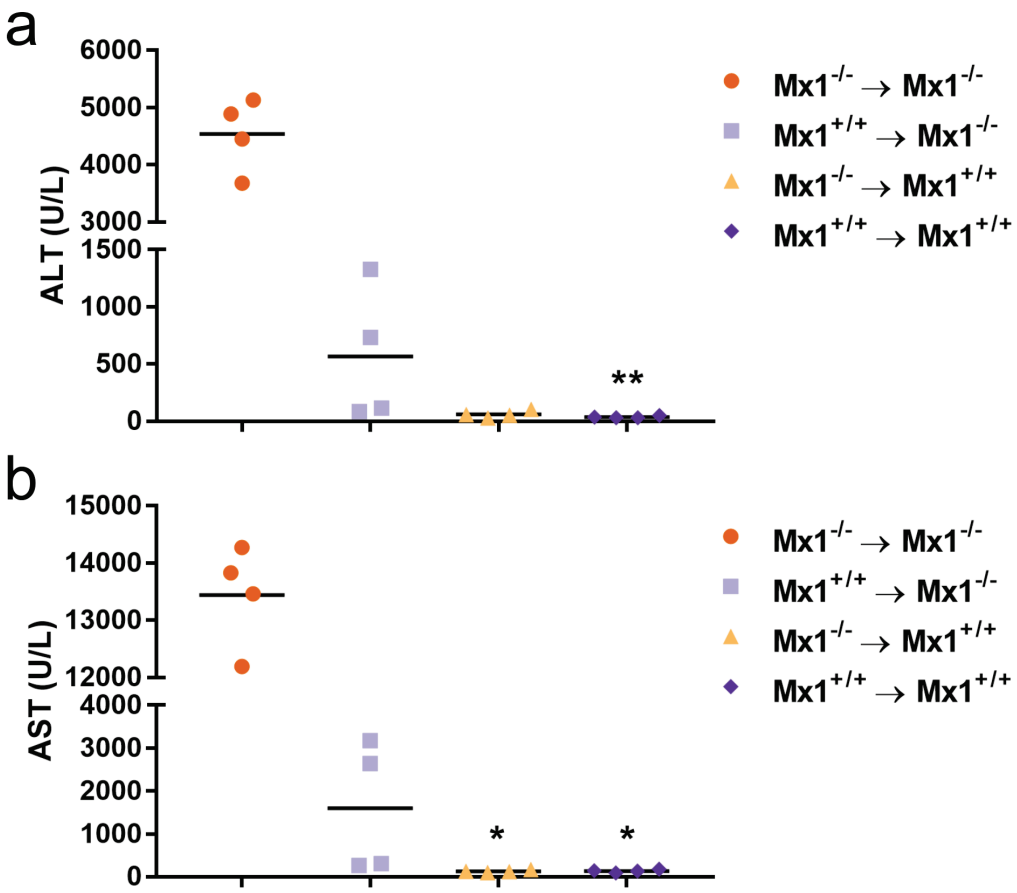

Figure 4.10 ALT and AST serum levels after THOV infection. Bone marrow chimeric mice were infected intraperitoneally with 1000 PFU of THOV. Four days after infection blood was drawn from the mice, and serum was prepared. Serum concentrations of ALT **(a)** and AST **(b)** were determined. Each data point represents the ALT or AST serum level of a single animal. Data are obtained in one single experiment. Asterisks indicate the significant difference with the Mx1$^{-/-}$ → Mx1$^{-/-}$ group. *, $p < 0.05$; **, $p < 0.01$; Kruskal-Wallis test with *post hoc* Dunn's multiple comparison test.

In order to quantify the amount of liver damage caused by THOV infection, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined in serum of THOV-infected bone marrow chimeric mice at 4 dpi. ALT and AST are enzymes which catalyze the transfer of α-amino groups from alanine and aspartate, respectively, to the α-ketogroup of ketoglutaric acid. Hereby, ALT and AST respectively generate pyruvic and oxalacetic acid which are important for the citric acid cycle. Both enzymes are highly concentrated in the liver, however, AST is also expressed in the heart, skeletal muscle, kidneys, brain, and in red blood cells. ALT can also be found in skeletal muscle and kidneys, albeit at low concentrations. Liver injury (acute or chronic) causes elevation of serum concentrations of ALT and AST because damaged liver cells 'spill' these enzymes into the bloodstream. Since ALT is more confined to the liver in comparison to AST, it is considered to be more specific for liver injury than AST[29]. The serum levels of both ALT and AST increase markedly in THOV-infected Mx1$^{-/-}$ → Mx1$^{-/-}$ mice in comparison to Mx1$^{-/-}$ →

$Mx1^{+/+}$ and $Mx1^{+/+}$ → $Mx1^{+/+}$ mice (Figure 4.10). The $Mx1^{+/+}$ → $Mx1^{-/-}$ group shows intermediate levels of both enzymes indicating that liver injury is less severe in these animals compared to the $Mx1^{-/-}$ → $Mx1^{-/-}$ group. This is in accordance with the histological scoring of the liver sections, further substantiating that $Mx1^{+/+}$ → $Mx1^{-/-}$ mice are better protected against the effects of THOV infection than $Mx1^{-/-}$ → $Mx1^{-/-}$ mice.

4.3 Discussion

In the mouse model, it is well established that expression of a functional Mx1 protein can protect against a lethal FLUAV or THOV infection, whereas lack of a functional Mx1 protein leads to severe morbidity and mortality in these models. *Mx1* is an interferon-stimulated gene, so it mainly acts as a part of the innate immune response by early inhibition of the virus in infected epithelial cells. However, until today the mechanism by which Mx1 protects these animals against infection remains elusive. In theory, every cell type that can mount a type I or III IFN response is also able to express Mx1 protein. Does this mean that, in response to viral infection, every cell type expresses Mx1, or that Mx1 is needed for their proper functioning? In this respect, several studies have shown the importance of an IFN response in cell types involved in the antiviral immune response after FLUAV infection[21-23]. These studies make it tempting to hypothesize that Mx1 can also play a role in the immune cell compartment, or better said, in the protection of cell types which are involved in the antiviral immune response. However, studies that concluded that Mx1 fulfills no function in the immune cell compartment only focused on a certain part of this compartment[17-19]. To our knowledge, the possible role of Mx1 in immune cells has not yet been studied to the full. To tackle this question, we generated bone marrow chimeras, wherein $Mx1^{-/-}$ recipient mice received $Mx1^{+/+}$ bone marrow, and *vice versa*. These animals were then used in several infection experiments. This would give us the chance to investigate the effect of Mx1 when it is primarily expressed by bone marrow-derived cells or stromal cells. However, one should take into consideration that some bone marrow-derived cell types, such as Langerhans cells[30] and mesenchymal stromal cells (reviewed by Sugrue *et al.*[31]), are resistant to lethal total body irradiation. Consequently, these cell types will have the genotype of the bone marrow recipient, and not that of the bone marrow donor. Since these cell types are not directly involved in the immune responses which were investigated, this is of minor importance for the obtained results.

FLUAV infection of bone marrow chimeras led to significantly more body weight loss and higher lung viral titers in the $Mx1^{-/-}$ recipients ($Mx1^{-/-}$ → $Mx1^{-/-}$ and $Mx1^{+/+}$ → $Mx1^{-/-}$) compared to the $Mx1^{+/+}$ recipients ($Mx1^{-/-}$ → $Mx1^{+/+}$ and $Mx1^{+/+}$ → $Mx1^{+/+}$). In viral mRNA and protein levels in the lung the same divergence was observed between $Mx1^{-/-}$ and $Mx1^{+/+}$ recipients. Remarkably, strong Mx1 expression was observed for the $Mx1^{+/+}$ → $Mx1^{-/-}$ mice,

which only express a functional Mx1 in their cells from hematopoietic origin. This is probably due to a high influx of immune cells into the lung, and a strong IFN response as a result of the FLUAV infection. These data suggest that resistance to infection is primarily dependent on the genotype of the recipient, and not so much on the genotype of the donor bone marrow.

In order to determine whether Mx1 has a direct or indirect effect on a specific immune cell type, we measured the levels of different cell types in the lung by flow cytometry at different time points prior to (0 dpi) or after infection (3 and 6 dpi). The influx of moDCs is nearly twofold higher in $Mx1^{-/-}$ than in $Mx1^{+/+}$ recipients. This probably indicates that the inflammatory response in $Mx1^{-/-}$ recipients is higher than in $Mx1^{+/+}$ recipients, since upon FLUAV infection lung inflammation causes recruitment of monocytes to the lung which then differentiate into moDCs[25]. This difference in inflammation also correlates with the differences seen for the expression of IFN-induced protein IFITM3. AMs and eosinophils get depleted, but this depletion is less strong in the $Mx1^{+/+}$ recipients than is the case for the $Mx1^{-/-}$ recipients. Alveolar macrophages die after taking up infected or dying cells, and virus particles[25,26]. Eosinophils are usually not recruited to the lung after FLUAV infection[32], and in addition, they are susceptible to this infection[33], which might explain why they get depleted faster in mice which do not express Mx1 in their bone marrow-derived cell types.

During the first six days after FLUAV infection no clear differences were seen in the T cell levels between all chimeric groups. We reexamined the antigen-specific T cell compartment at ten days post infection. Although there is a clear antigen-specific T cell response in all chimeric groups, we observed no significant differences between the $Mx1^{-/-} \rightarrow Mx1^{-/-}$ and the $Mx1^{+/+} \rightarrow Mx1^{-/-}$ groups, nor between the $Mx1^{-/-} \rightarrow Mx1^{+/+}$ and the $Mx1^{+/+} \rightarrow Mx1^{+/+}$ groups. This indicates that Mx1 expression in bone marrow-derived cells is not important for the formation of an efficient antigen-specific T cell response. More importantly however, these results show that $Mx1^{+/+}$ recipients can form an adequate antigen-specific T cell response after FLUAV infection.

Taken together, the data gathered with this FLUAV infection model do not answer the question if Mx1 can play a role in the immune cell compartment. Maybe this infection model is not the most eligible for answering this question. Therefore we substituted the FLUAV with another virus, *i.e.* THOV. The rationale for choosing this virus is threefold: THOV, like influenza A virus, is a member of the Orthomyxovirus family, it is also sensitive to the antiviral action of murine Mx1[28], and small rodents are natural hosts of this virus[5].

Bone marrow chimeras were infected intraperitoneally with a high dose of THOV. Morbidity was somewhat different than seen in the FLUAV infection model in that the $Mx1^{+/+} \rightarrow Mx1^{-/-}$ group shows less body weight loss than the $Mx1^{-/-} \rightarrow Mx1^{+/+}$ group. Remarkably, liver viral titers at 4 dpi were very high for all the $Mx1^{-/-}$ recipients, and below the detection limit for $Mx1^{+/+}$ recipients. This is contradictory to the morbidity data where $Mx1^{+/+} \rightarrow Mx1^{-/-}$ mice seem to lose less body weight after infection than the mice from the $Mx1^{-/-} \rightarrow Mx1^{+/+}$ group.

When examining Mx1 expression in the liver, we noticed that the $Mx1^{-/-} \rightarrow Mx1^{+/+}$ group showed very high Mx1 protein levels. The Mx1 protein levels are much lower in the $Mx1^{+/+} \rightarrow Mx1^{-/-}$ and the $Mx1^{+/+} \rightarrow Mx1^{+/+}$ groups. Especially the latter is remarkable, because in the FLUAV infection model we also observed a level of Mx1 expression in the $Mx1^{+/+} \rightarrow Mx1^{+/+}$ group comparable to that in the $Mx1^{-/-} \rightarrow Mx1^{+/+}$ group. This discrepancy is likely caused by the lack of an IFN response in the livers of $Mx1^{+/+} \rightarrow Mx1^{+/+}$ mice, which is demonstrated by the lack of ISG15 expression in this group. Presumably, the IFN response is triggered by a productive THOV infection in the liver. A high level of ISG15 expression is seen for the $Mx1^{-/-} \rightarrow Mx1^{-/-}$ group, which also shows high levels of THOV NP expression. In the $Mx1^{+/+} \rightarrow Mx1^{-/-}$ group we observed little to no THOV NP expression which is surprising seeing that mice from this group showed high liver viral titers. In this $Mx1^{+/+} \rightarrow Mx1^{-/-}$ group the highest ISG15 expression is seen in the animals which show some THOV NP expression. Conversely, the $Mx1^{-/-} \rightarrow Mx1^{+/+}$ group, which showed a high Mx1 expression, also shows ISG15 expression, but no THOV NP expression at all. In contrast, the $Mx1^{+/+} \rightarrow Mx1^{+/+}$ group also shows no THOV NP expression in the liver, but also no ISG15 and very little Mx1 expression.

So far, the data obtained with the THOV infection model are quite puzzling. In order to find an explanation for the seemingly discrepant data, we examined the liver tissue of THOV-infected bone marrow chimeras on a macro- and microscopic level. On microscopic scale, both $Mx1^{-/-} \rightarrow Mx1^{-/-}$ and $Mx1^{+/+} \rightarrow Mx1^{-/-}$ liver tissue showed lesions. As a more objective measure for liver damage, we measured the serum levels of two enzymes, ALT and AST. The obtained data reflected the results of the histological analysis of the liver tissue. Taken together, these results suggest that the expression of a functional Mx1 protein in hematopoietic-derived cells cannot protect against productive THOV infection in $Mx1^{-/-}$ recipients, but it can delay its progression.

Recently, Kochs *et al.* postulated that THOV has a clear tropism for $CD11b^{+}$ cells with a clear myeloid/macrophage phenotype (double positive for surface markers CD11b and F4/80) in the peritoneum[34]. This could explain the obtained data, and allows us to come up with a

hypothesis about the role of Mx1 in this THOV infection model. Seeing that THOV shows a tropism for myeloid cells in the peritoneum, it is conceivable that – when these myeloid cells express Mx1 – they are (partially) protected against THOV infection. If these cells do get infected by THOV, they probably transport the virus to the liver (Georg Kochs, personal communication). This is a credible theory seeing that Ghosn *et al.* identified a population of large peritoneal macrophages (LPMs) which seem to have a similar phenotype as the $CD11b^+$ myeloid cells described by Kochs *et al.*[34,35]. It has been shown that these LPMs migrate to the omentum – a fat tissue that connects the abdominal organs – upon inflammation[36], which is in agreement with the disappearance of the $CD11b^+$ myeloid cell population from the peritoneal cavity after THOV infection[34]. By migrating to the omentum, the LPMs are also able to reach the liver of infected mice. Here, the virus could infect hepatocytes, unless these hepatocytes express a functional Mx1 protein. Viral replication would be blocked in the hepatocytes which would explain why $Mx1^{+/+}$ → $Mx1^{+/+}$ mice show no detectable virus titers, since they express Mx1 both in their stromal and their myeloid cells. $Mx1^{-/-}$ → $Mx1^{-/-}$ mice show no resistance against THOV, possibly because they do not express Mx1 in any cell type. In $Mx1^{+/+}$ → $Mx1^{-/-}$ mice, which only express Mx1 in their myeloid cells, we observed high viral titers and necrotic cell lesions in the liver, indicating that THOV can still reach the liver. However, THOV NP expression in the liver was low to nonexistent. It is plausible that myeloid cells are only partially protected against THOV infection, or that the viral inoculum (1000 PFU) is too high for the $Mx1^{+/+}$ myeloid cells. However, the obtained results indicate that Mx1 expression in myeloid cells can delay the progression of THOV infection. $Mx1^{-/-}$ → $Mx1^{+/+}$ mice showed no detectable liver viral titers and no liver injury. If THOV could reach the liver tissue via THOV-susceptible $Mx1^{-/-}$ myeloid cells, the virus would get inhibited by the presence of a functional Mx1 protein in the hepatocytes. However, this does not explain the stronger weight loss and higher Mx1 protein levels for these mice in comparison with the $Mx1^{+/+}$ → $Mx1^{-/-}$ and $Mx1^{+/+}$ → $Mx1^{+/+}$ groups. Conceivably, since THOV can still reach the liver quite easily in $Mx1^{-/-}$ → $Mx1^{+/+}$ mice, an inflammatory response will be triggered. This is demonstrated by the ISG15 expression in the livers of $Mx1^{-/-}$ → $Mx1^{+/+}$ mice. This response can cause the production of inflammatory cytokines[37,38], and probably is the reason for the high Mx1 expression and more severe weight loss in the $Mx1^{-/-}$ → $Mx1^{+/+}$ group.

In conclusion, to confer resistance against Mx1-susceptible viruses which do not have a tropism for myeloid cells, like influenza A/Puerto Rico/8/34, *Mx1* expression is only important in the stromal cells. However, for resistance against Mx1-susceptible viruses that infect myeloid cells, like THOV which has a tropism for $CD11b^+$ myeloid cells, *Mx1* expression in bone marrow-derived cells is of major importance. Nevertheless, *Mx1*

expression in stromal cells remains crucial for resistance, also against viruses with a tropism for myeloid cells.

4.4 Materials and methods

Ethics statement. All animal experiments described in this study were conducted according to the national (Belgian Law 14/08/1986 and 22/12/2003, Belgian Royal Decree 06/04/2010) and European legislation (EU Directives 2010/63/EU, 86/609/EEC). All experiments on mice and animal protocols were approved by the ethics committee of Ghent University (permit numbers LA1400091 and EC2015-027).

Mice. Mice were bred in-house under Specific Pathogen Free (SPF) conditions. Mice were housed in individually ventilated cages, in a temperature-controlled environment with 12h light/dark cycles, with food and water *ad libitum*. B6.A2G Mx1 ($Mx1^{+/+}$) mice carrying a functional A2G *Mx1* allele (first described by Horisberger *et al.*[39]) were kindly provided by Peter Stäheli (University of Freiburg, Germany). Congenic B6.A2G Mx1 ($Mx1^{-/-}$) carrying the defective C57BL/6J *Mx1* allele were generated in our laboratory by crossing B6.A2G Mx1 ($Mx1^{+/+}$) with C57BL/6J ($Mx1^{-/-}$) mice, and subsequent crossing of the heterozygous offspring.

Bone marrow chimera mice. B6.A2G-Mx1 congenic mice were bred in-house under Specific Pathogen Free (SPF) conditions. Starting one week before until three weeks after irradiation, mice were given water containing 0.2% neomycin *ad libitum*. Mice were given lethal total body irradiation (10 Grey) with an X-Rad 320 Biological Irradiator (Precision X-Ray (PXi), North Brandford, Connecticut, USA), and 24h later they were reconstituted with syngeneic or allogeneic bone marrow cells (8-10 x 10^6) that were harvested from femurs of age- and sex-matched mice. Experimental transfers were as follows: B6.A2G $Mx1^{-/-}$ donors into B6.A2G $Mx1^{-/-}$ recipients ($Mx1^{-/-}$ → $Mx1^{-/-}$), B6.A2G $Mx1^{-/-}$ donors into B6.A2G $Mx1^{+/+}$ recipients ($Mx1^{-/-}$ → $Mx1^{+/+}$), B6.A2G $Mx1^{+/+}$ donors into B6.A2G $Mx1^{+/+}$ recipients ($Mx1^{+/+}$ → $Mx1^{+/+}$), and B6.A2G $Mx1^{+/+}$ donors into B6.A2G $Mx1^{-/-}$ recipients ($Mx1^{+/+}$ → $Mx1^{-/-}$). Animals were allowed to reconstitute for eight weeks.

Virus challenge. Mice were challenged with 10 LD_{50} (approximately 170 PFU) of mouse adapted (ma) influenza A/Puerto Rico/8/34 (PR8) (H1N1) or with 10^3 PFU Thogoto virus SiAr 126 (THOV) (Kindly provided by Georg Kochs (University of Freiburg, Germany)). The challenge dose was administered intranasal in a volume of 50 µl (maPR8) or intraperitoneally in a volume of 100 µl (THOV) to mice anesthetized with a mixture of ketamine (10 mg/kg) and xylazine (60 mg/kg). Morbidity was monitored during six (maPR8) or four (THOV) days post infection. Loss of 25% of bodyweight was used as a humane endpoint for euthanizing moribund mice.

Determination of influenza lung virus titers. Several mice of each group were killed at different time points (0, 3, and 6 days post infection (dpi)) by intraperitoneal injection of pentobarbital (125 µg/g). The mouse lungs were removed aseptically, and the left lobe was snap-frozen in liquid nitrogen until further processing. Lung extracts were made by homogenizing the lungs in PBS using metal beads. Cell debris was cleared by centrifugation for 10 min at 400g and 4°C. Cleared lung homogenates were stored at -80°C. Influenza virus titers were determined in triplicate by plaque titration on MDCK cells (300 000 cells per well in a 12-well plate). Monolayers were infected for 1h with 500 µl of serial 1:10 dilutions of the lung homogenates in serum-free DMEM medium supplemented with penicillin and streptomycin. Following inoculation, the supernatant was replaced by medium containing 2 µg/ml trypsin and 0.6% avicel RC-851 (FMC Biopolymers). Two days after infection, cells were fixed with 4% paraformaldehyde, and permeabilized with PBS containing 0.2% Triton X-100. Plaques were stained using a mouse monoclonal antibody against the ectodomain of the influenza M2 protein (mAb65[40]), and an HRP-conjugated anti-mouse IgG antibody (Sheep anti-mouse IgG HRP, GE Healthcare, UK). Plaques were then visualized by using TrueBlue peroxidase substrate (Seracare, Gaithersburg, MD, USA).

Determination of Thogoto liver virus titers. Mice were killed by cervical dislocation, the liver was removed aseptically, and one of the lobes was used for histochemistry. Of the remaining tissue, extracts were made by homogenizing the livers in PBS using metal beads. Cell debris was cleared by centrifugation for 10 min at 400g and 4°C. Cleared liver extracts were stored at -80°C. Thogoto virus titers were determined in duplicate by titration on Vero cells. Monolayers (300 000 cells in a 6-well plate) were infected for 1h with 1 ml of serial 1:10 dilutions of the liver homogenates in DMEM medium supplemented with 2% fetal calf serum and 20 mM HEPES pH 7.3. Following inoculation, the supernatant was replaced by medium containing 0.6% avicel RC-951 (FMC Biopolymers). Four days after infection, cells were fixed with 4% paraformaldehyde. The cell monolayers were stained with a crystal violet solution (1% crystal violet + 1% methanol + 20% ethanol) for approximately 15 minutes at room temperature. The crystal violet solution was removed, and monolayers were washed with water.

Histopathological examination of livers. Livers of bone marrow chimeric mice (n = 4 per group) were excised at 4 dpi. After fixation in 4% paraformaldehyde (PFA) and embedding in paraffin, livers were sectioned at 5 µm and stained with hematoxylin and eosin for histological evaluation. Images were obtained with an Axioscan.Z1 slide scanner (Zeiss, Oberkochen, Germany), and were analyzed with ZEN Lite software (Zeiss, Oberkochen, Germany).

ALT/AST assay. Blood was taken via retro-orbital bleeding after sedation of the mice with pentobarbital (125 µg/g). To prepare serum, samples were let to clot overnight at 4°C. The next day, samples were centrifuged at 14000 rpm for 3 minutes and obtained serum samples were stored at -20°C. Levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using a Hitachi kit and apparatus in the Clinical Biology Laboratory of Ghent University Hospital.

Flow cytometry. Several mice of each group were killed at different time points (0, 3, and 6 dpi) by intraperitoneal injection of pentobarbital (125 µg/g). The mouse lungs and mediastinal lymph nodes were removed aseptically; only the right lobes of the lung were used for flow cytometric analysis. Lung single cell suspensions were generated using 70 µm nylon cell strainers (Falcon, Corning, NY, USA). The immune cell composition of these tissues was then determined by analyzing the surface expression of different lineage markers. Briefly, high-affinity Fc receptors (FcRs) were blocked by incubation with purified anti-mouse CD16/CD32 (Fc Block, BD Pharmingen, 553142, 1/500) for 30 min at 4°C. Subsequently the cells were stained with an antibody panel designed to specifically analyze the T cell composition or with a broader panel for 1h at 4°C in the dark. For the T cell panel, cells were stained with a fixable viability stain (eBioscience Fixable Viability Dye eFluor 506, ThermoFischer, 65-0866-14, 1/1000), CD3e-PE (eBioscience, ThermoFisher, 12-0031-81, 1/300), CD4-PE-Cy5 (BD Pharmingen, 561836, 1/300), MHCII-PerCP-Cy5.5 (BioLegend, 107625, 1/500), CD8a-PE-Cy7 (eBioscience, ThermoFisher, 25-0081-81, 1/300), and CD45-AF700 (eBioscience, ThermoFisher, 56-0451-82, 1/300). For the broader panel, cells were stained with a fixable viability stain (eBioscience Fixable Viability Dye eFluor 450, ThermoFischer, 65-0863-14, 1/1000), XCR1-BV510 (BioLegend, 148218, 1/400), CD11b-BV605 (BD Horizon, 563015, 1/500), CD64-BV711 (BioLegend, 139311, 1/100), SiglecF-PE (BD Pharmingen, 552126, 1/1000), CD172a-Biotin (eBioscience, ThermoFischer, 13-1721-82, 1/100), Streptavidin-PE-CF594 (BD Horizon, 562284, 1/1000), CD3-PE-Cy5 (eBioscience, ThermoFischer, 15-0031-82, 1/200), CD19-PE-Cy5 (eBioscience, 15-0193-83, 1/200), CD11c-PE-Cy7 (eBioscience, ThermoFischer, 25-0114-82, 1/800), MHCII-APC-eFluor780 (eBioscience, ThermoFisher, 47-5321-82, 1/1500), and CD45-AF700 (eBioscience, ThermoFisher, 56-0451-82, 1/300). After staining, samples were analyzed on an LSRFortessa flow cytometer (BD Biosciences, San Jose, CA, USA), and analyzed using FlowJo X (Treestar) software.

Intracellular cytokine staining (ICS) and flow cytometry after *ex vivo* restimulation. Ten days after FLUAV infection, spleens were isolated aseptically and splenocytes were prepared. After lysis of RBCs with NH_4Cl solution, 5 x 10^5 splenocytes were plated in 1 ml of culture medium (RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-

glutamine, 0.4 mM Na-pyruvate, nonessential amino acids, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.) supplemented with $NP_{366-374}$ ASNENMETM peptide (Genscript, RP20267-5) at 4 µg/ml. After 6h of peptide restimulation, 1 µl Golgiplug (brefeldin A, BD, Erembodegem, Belgium) was added to 1 ml culture medium for measurement of cytokine production by ICS. The Cytofix/Cytoperm kit (BD) was used according to the manufacturer's protocol. Briefly, 16h after addition of Golgiplug, cells were stained with a Live/Dead marker (eBioscience Fixable Viability Dye eFluor 450, 65-0866-14, ThermoFisher, 1/1000), and with fluorochrome labeled antibodies against MHCII (MHCII-eFluor450, 48-5321-82, ThermoFischer, 1/500), CD3 (CD3-AF488, 557666, BD Pharmingen, 1/250), CD4 (CD4-PE-Cy5, BD Pharmingen, 561836, 1/300) and CD8 (CD8-PE-Cy7, 25-0081-81, ThermoFischer, 1/300). Cells were then fixed/permeabilized using the Cytofix/Cytoperm kit (BD), and stained for IFNγ (IFNγ-APC, BD Pharmingen, 554413, 1/100). Cells were then analyzed by using an LSR Fortessa (BD) with FlowJoX software (Treestar, Ashland, Orlando).

Real-time quantitative PCR (RT-qPCR). Cleared lung homogenates obtained from infected mice, as described for the determination of influenza lung virus titers, were stored at -80°C. RNA isolation was done with the High Pure RNA Isolation Kit (11828665001, Roche) as indicated by the manufacturer. Total mRNA was converted to cDNA by RT-PCR using oligo-dT reaction (Transcriptor First Strand cDNA Synthesis Kit, 04897030001, Roche). Ten nanogram of cDNA was used for each reaction, and triplicate reactions were setup in 384-well plates. qPCR reactions based on SYBR green detection, were performed using a LightCycler equipment (Roche). qPCR-data were analyzed using the qbase+ software packet (Biogazelle, Zwijnaarde, Belgium).

List of primers used in this study: 5'-GGGAAGAACACCGATCTTGA-3'; M1/2 reverse: 5'-CGGTGAGCGTGAACACAAAT-3'; NA forward: 5'-CATCTCTTTGTCCCATCCGT-3'; NA reverse: 5'-GTCCTGCATTCCAAGTGAGA-3'; HA forward: 5'-GAGGAGCTGAGGGAGCAAT-3'; HA reverse: 5'-GCCGTTACTCCGTTTGTGTT-3'; PB1 forward: 5'-CCTCCTTACAGCCATGGGA-3'; PB1 reverse: 5'-GTGCTCCAGTTTCGGTGTTT-3'; PB2 forward: 5'-GGATCAGACCGAGTGATGGT-3'; PB2 reverse: 5'-CCATGCTTTAGCCTTTCGACT-3'; PA forward: 5'-CATCAATGAGCAAGGCGAGT-3'; PA reverse: 5'-GCCCCTGTAGTGTTGCAAAT-3'; NP forward: 5'-CAGCCTAATCAGACCAAATG-3'; NP reverse: 5'-TACCTGCTTCTCAGTTCAAG-3'; NS1 forward: 5'-TTCACCATTGCCTTCTCTTC-3'; NS1 reverse: 5'-CCCATTCTCATTACTGCTTC-3'; HPRT1 forward: 5'-AGTGTTGGATACAGGCCAGAC-3'; HPRT1 reverse: 5'-CGTGATTCAAATCCCTGAAGT-3'; UBC forward: 5'-AGGTCAAACAGGAAGACAGACGTA-3'; UBC reverse: 5'-TCACACCCAAGAACAAGCACA-3'; GAPDH forward: 5'-TGAAGCAGGCATCTGAGGG-3'; GAPDH reverse: 5'-

CGAAGGTGGAAGAGTGGGAG-3'; TBP forward: 5'-TCTACCGTGAATCTTGGCTGTAAA-3'; TBP reverse: 5'-TTCTCATGATGACTGCAGCAAA-3'; RPL13A forward: 5'-CCTGCTGCTCTCAAGGTT-3'; RPL13A reverse: 5'-TGGTTGTCACTGCCTGGTACTT-3'; actin forward: 5'-GCTTCTAGGCGGACTGTTACTGA-3'; actin reverse: 5'-GCCATGCCAATGTTGTCTCTTAT-3'.

Antibodies used in western blot analysis. A polyclonal antiserum against mouse Mx1 was generated by immunizing New Zealand White rabbits three times subcutaneously with 100 µg of the synthetic, high-performance liquid chromatography-purified peptide CKKFLKRRLLRLDEARQKLAKFSD (C terminus of the Mx1 protein) combined with the water-in-oil adjuvant Montanide ISA-720 (SEPPIC SA, Paris, France). The serum IgG fraction was enriched by 50% ammonium sulphate precipitation followed by affinity chromatography with a protein A column (GE Healthcare). M2e-specific monoclonal antibody was produced in our laboratory. Briefly, hybridomas that produce M2e-specific monoclonal antibodies were isolated described (Cho et al., 2015, J Virol). After subcloning, these hybridoma cultures were scaled up and monoclonal antibodies were purified from the culture supernatant with a protein A column (GE Healthcare). Polyclonal anti-influenza virus RNP antibody, A/Scotland/840/74 (H3N2), (antiserum, goat), NR-3133 was obtained from the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH. Rabbit polyclonal anti-mouse IFITM3 antibody was obtained from Abcam (ab15592). Monoclonal anti-actin antibody (mouse) was purchased from MP Biomedicals (08691001). Polyclonal anti-Thogoto virus NP antibody, (antiserum, rabbit), was kindly provided by Georg Kochs (University of Freiburg, Germany). Monoclonal anti-ISG15 antibody (mouse) was obtained from Santa Cruz Biotechnology Inc. (sc-166755).

Statistical analysis. The obtained data were analyzed using Graphpad Prism 7 or Genstat software. Methods used in Genstat are described below. Statistical tests performed in Graphpad Prism 7 software are mentioned in figure legends. In Figure 4.7a we used a two-way ANOVA test with *post hoc* Tukey's HSD test. This test is used to compare multiple groups when multiple variables are at play. Parametric tests did not apply for Figures 4.6, 4.7b, 4.10 and S4.3 according to the D'Agostino-Pearson omnibus normality test due to the small number of samples. Therefore, the non-parametric alternatives were used. In Figure 4.6 a two-sided Mann-Whitney U test was used, this is the non-parametric alternative for a two-sided T test. In Figures 4.7b, 4.10 and S4.3 we used the Kruskal-Wallis test (the non-parametric alternative for a one-way ANOVA test) with *post hoc* Dunn's multiple comparison test.

Relative body weight data analysis

Relative body weight data was analyzed as repeated measurements using the residual maximum likelihood (REML) approach as implemented in Genstat v18 (Payne, 2015). Briefly, a linear mixed model with replicate, genotype, time and genotype x time interaction as fixed terms, and subject.time used as residual term, was fitted to data. Times of measurement were set at equal intervals and an autoregressive correlation structure of order 1 with equal variances (i.e. homogeneity across time) was selected as best model fit in all cases, based on the Aikake Information Coefficient. Significances of the fixed terms and significances of changes in differences between genotype effects over time were assessed by an F-test.

Analysis of virus titers

A linear fixed model with replicate, genotype, time and genotype x time interaction was fitted to the data. Significances of the fixed terms and significances of changes in differences between genotype effects over time were assessed by an F-test.

Analysis of flow cytometry data

A linear fixed model with replicate, genotype, time and genotype x time interaction as fixed terms was fitted to the eosinophil, neutrophil, $CD4^+$ T cell, $CD8^+$ T cell, AM, moDC, cDC1 and cDC2 cells counts expressed as a proportion of $CD45^+$ cells. Plots of residuals against fitted values indicated homogeneity of variances in all cases, excluding transformation of the data. Significances of the fixed terms and significances of changes in differences between genotype effects over time were assessed by an F-test.

Analysis of RT-qPCR data

A Generalized Linear Mixed Model (GLMM) (fixed model: poisson distribution, log link; random model: gamma distribution, log link) as implemented in Genstat v18[41] has been fitted to RT-qPCR expression data of PB1, PB2, PA, NP, HA, NA, M and NS genes simultaneously. The linear predictor vector of the values can be written as follows: $\log(\mu) = \eta = X\beta + Z\nu$, where the matrix X is the design matrix for the fixed terms genotype, time and genotype × time, β is their vector of regression coefficients, Z is the design matrix for the random term (i.e. gene, replicate and gene × replicate), and ν is the corresponding vector of random effect having a gamma distribution. The significance of the fixed interaction term genotype × time was assessed by a Wald test. Significance of the regression coefficients were assessed by a *t*-test. Estimated mean values and their standard errors were obtained as predictions from the GLMM, formed on the scale of the response variable.

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Supplementary figures

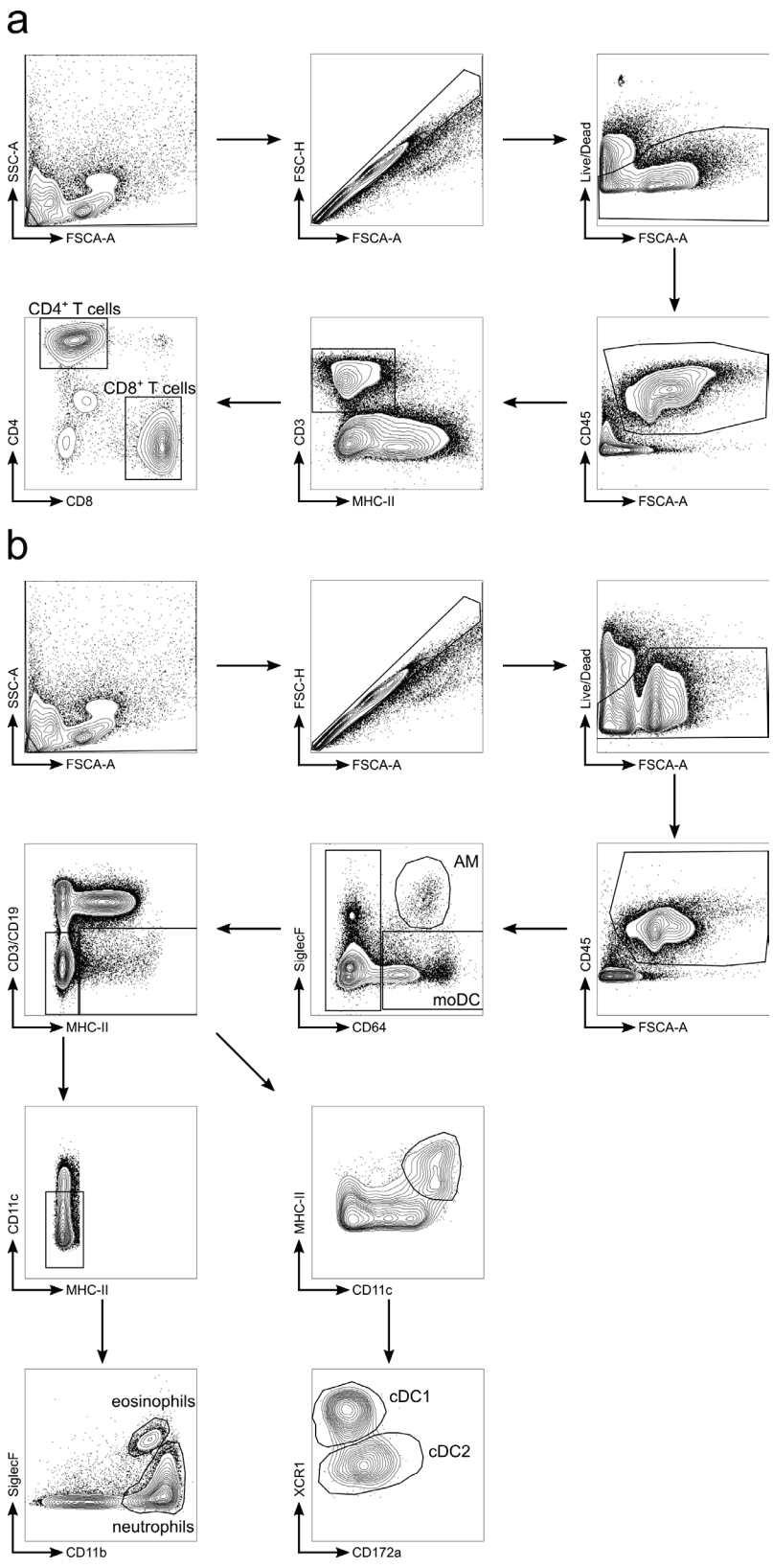

Figure S4.1 Gating strategy of flow cytometry analysis. Gating strategy of the T cell panel **(a)** and the broader panel **(b)**. AM = alveolar macrophage, moDC = monocyte-derived dendritic cell, cDC = conventional dendritic cell.

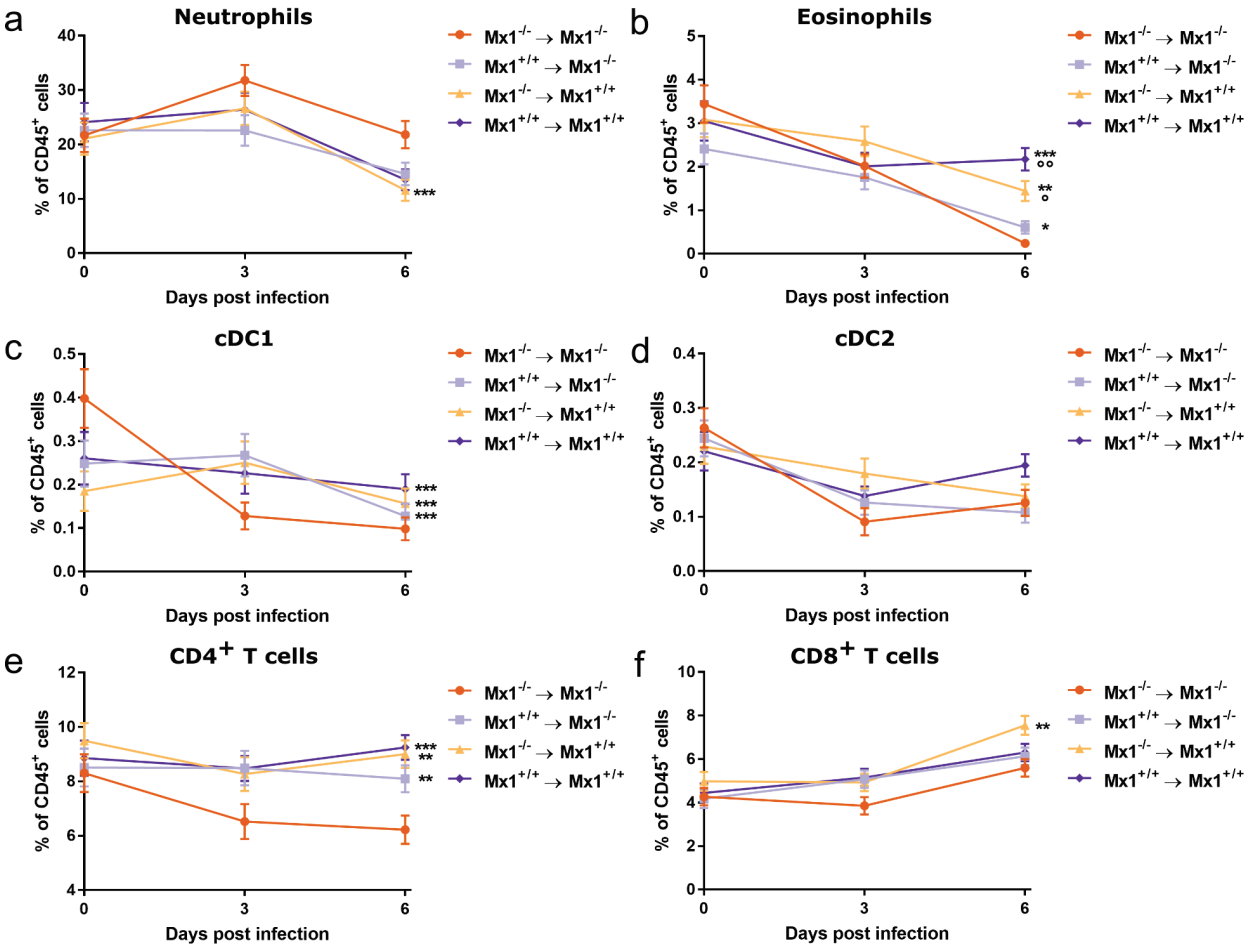

Figure S4.2 Comparison of different cell types over time between four bone marrow chimeric groups after FLUAV infection. Bone marrow chimeric mice were infected with 10 LD_{50} of maPR8, and lungs were isolated at 0, 3 and 6 dpi. These lungs were processed to single cell suspensions, which were stained for different surface markers. This allowed us to discriminate neutrophils **(a)**, eosinophils **(b)**, conventional dendritic cells type 1 **(c)**, conventional dendritic cells type 2 **(d)**, $CD4^+$ T cells **(e)**, and $CD8^+$ T cells **(f)**. Data points represent the average level of at least six mice. Error bars represent standard error of the mean. Asterisks indicate the significant difference with the $Mx1^{-/-}$ → $Mx1^{-/-}$ bone marrow chimera group over time. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Circles indicate the significant difference with the $Mx1^{+/+}$ → $Mx1^{-/-}$ bone marrow chimera group over time. °, $p < 0.05$; °°, $p < 0.01$. Data are pooled from 2 independently performed experiments.

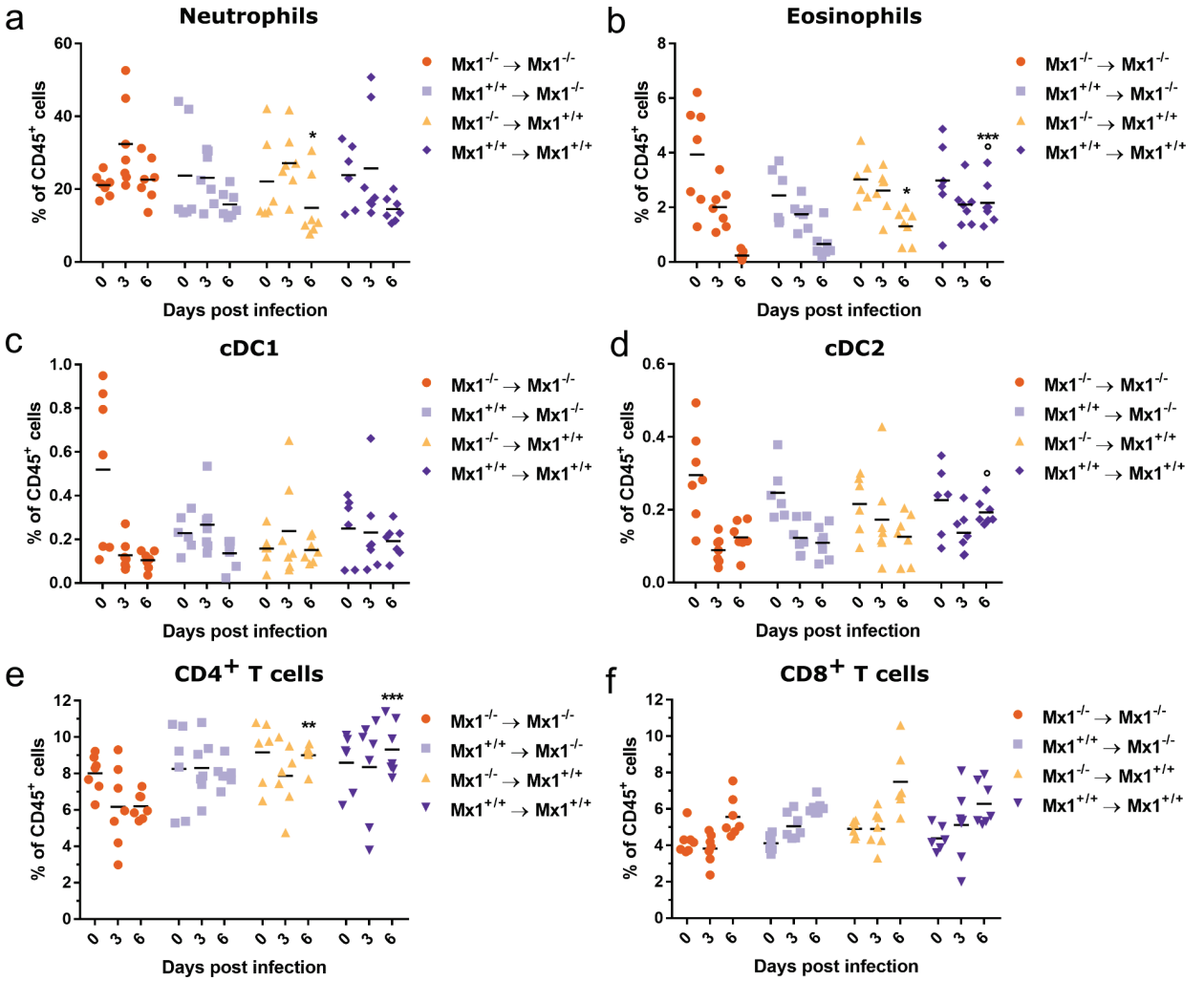

Figure S4.3 Comparison of different cell types between four bone marrow chimeric groups after FLUAV infection. Same data as in Figure 4.5 and S2, showing the individual level of monocyte-derived DCs **(a)**, alveolar macrophages **(b)**, neutrophils **(c)**, eosinophils **(d)**, conventional dendritic cells type 1 **(e)**, conventional dendritic cells type 2 **(f)**, $CD4^+$ T cells **(g)**, and $CD8^+$ T cells **(h)**. Each data point represents the relative amount of cells of each indicated cell type of a single animal. Asterisks indicate the significant difference with the $Mx1^{-/-}$ → $Mx1^{-/-}$ bone marrow chimera group at the indicated time point. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Circles indicate the significant difference with the $Mx1^{+/+}$ → $Mx1^{-/-}$ bone marrow chimera group at the indicated time point. °, $p < 0.05$; °°, $p < 0.01$. Kruskal-Wallis test with *post hoc* Dunn's multiple comparison test. Data are pooled from 2 independently performed experiments.

Mah Na Mah Na
Do do do do do
The Muppet Show

CHAPTER 5: Mx1 modulates the cellular influenza A virus nucleoprotein-specific immune responses induced by MVA-NP

Mx1 modulates the cellular influenza A virus nucleoprotein-specific immune responses induced by MVA-NP

Running title: Involvement of Mx1 in MVA-NP-induced immunogenicity

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JS and KR performed the experiments. JS, KR and XS designed the experiments. KR and XS supervised the research. JS, KR and XS co-wrote the manuscript.

Abstract

Current influenza vaccines are designed to mainly elicit strong antigen-specific humoral responses. This strategy proves to be very efficient when the vaccine strains match the circulating influenza strains. However, in case of a mismatch the vaccine efficacy is greatly reduced. To prevent this in the future, a lot of effort has already been put into the development of influenza vaccines which induce broadly protective and long-lasting immunity. Eliciting specific $CD8^+$ T cells directed against the influenza A virus (FLUAV) nucleoprotein (NP) is a frequently used strategy to do this. Therefore, Altenburg *et al.* used recombinant modified vaccinia Ankara (rMVA) as a vaccine vector for the induction of FLUAV NP-specific $CD8^+$ T cells. They introduced several modifications to NP to enhance the retention and degradation in the cytosol, and consequently increase antigen processing and presentation to improve the induction of a $CD8^+$ T cell response. These rMVA-NP constructs showed promising results *in vitro*, but displayed no differences with the wild type NP construct after vaccination of C57BL/6 mice. Here, we tested the rMVA-NP constructs in the B6.A2G $Mx1^{+/+}$ mouse strain, which expresses the Mx1 protein. This protein has a strong antiviral effect against FLUAV. Hence, we hypothesized that Mx1 could be an extra limiting factor for the different rMVA-NP constructs after vaccination. In this model, the modified rMVA-NP constructs would have an advantage over the wild type rMVA-NP construct. We showed that, although there is a trend that certain modified rMVA-NP constructs perform better than the wild type rMVA-NP construct, there are no significant differences between the different rMVA-NP constructs when used in the B6.A2G $Mx1^{+/+}$ mouse strain.

5.1 Introduction

Current influenza vaccines mainly elicit antibodies directed against the influenza HA , and only a limited cellular immune response[1,2]. For a cellular immune response to occur, host cells should produce viral peptides which they present on their cell surface by means of MHC molecules. These viral peptides are generated through processing of viral proteins by the proteasome located in the cytosol. These peptides are loaded onto MHC molecules which are transported to the cell surface, where they get recognized by T cells (MHC-I molecules are recognized by $CD8^+$ T cells, and MHC-II molecules are recognized by $CD4^+$ T cells)[3]. The bulk of FLUAV-specific $CD8^+$ T cells recognize NP-derived epitopes[4-6].

Modified vaccinia Ankara (MVA) is a Vaccinia virus-derived live attenuated virus that can efficiently induce T cell responses. MVA has been exploited as a vaccine that could protect against small pox virus, and as a recombinant vaccine vector to induce an immune response against heterologous antigens. MVA has a number of advantages making it an ideal system for delivery of viral antigen[7]. For example, the antigen of interest is expressed in its native conformation, and MVA can elicit high levels of antigen-specific humoral and cellular immune responses. The use of a live vector allows *de novo* synthesis of viral proteins in the cytosol of antigen-presenting cells which facilitates the antigen processing and presentation to $CD8^+$ T cells. The MVA-NP+M1 vaccine, which expresses the internal FLUAV proteins NP and M1, was designed to specifically induce virus-specific T cell responses. This vaccine was tested in clinical trials and showed mainly induction of $CD8^+$ T cells[8]. The MVA-NP+M1 vaccine was also able to protect against experimental infection one month after vaccination, and was shown to be safe for administration to elderly people[9,10].

Altenburg and colleagues hypothesized that the immunogenicity of NP expressed by an MVA vector could be increased by enhancing the cytosolic retention and degradation of NP. For this they modified the NLS of NP to prevent it from entering the nucleus. This was done in two different ways. Either two residues of the NLS were mutated into alanine residues (NPmut), or the NLS was completely deleted (NPΔNLS). Another way to alter the cytosolic degradation of NP was to fuse ubiquitin to the amino-terminus of the protein (UbqNP). This targets the fusion protein for degradation by the proteasome. Initial tests with recombinant MVA (rMVA) vectors expressing these different NP variants showed that the modifications had the intended effects *in vitro*. After infection with a low dose of the rMVA constructs, NP-specific $CD8^+$ T cell clones with either a low or a high functional avidity were both activated more efficiently by the mutated NP constructs than by the wild type NP construct. When infected with a high dose of the rMVA constructs, this effect was only apparent for the T cell clone with a low functional avidity, the high functional avidity T cell clone showed no more

differences in activation between the different NP constructs. This indicates that under suboptimal conditions (limited amounts of antigen or $CD8^+$ T cells of low functional avidity), modifying NP improves the $CD8^+$ T cell response *in vitro*. Moreover, pulse-labeling experiments allowed to conclude that the NP variants expressed by the rMVA-NPΔNLS and rMVA-UbqNP constructs were subject to accelerated degradation of NP in infected cells.

Immunization of C57BL/6 mice with these rMVA-NP constructs reduced body weight loss and viral replication in the lungs after a lethal challenge with influenza A/Puerto Rico/8/34 virus, but did not reveal differences between the constructs. The differential effects *in vitro*, and the lack of these effects *in vivo*, led us to the following hypothesis. In order to elicit a strong $CD8^+$ T cell response, the amount of processed antigen should reach a certain threshold. In C57BL/6 mice, this $CD8^+$ T cell response is dominated by $NP_{366-374}$-specific $CD8^+$ T cells, and this antigen-threshold is readily reached after vaccination with rMVA-NPwt[11]. Demonstrating the possible enhanced T cell responses against NP elicited by vaccination with vectored NP variants may therefore require a host in which NP-specific T cell responses are restricted at an additional level. We hypothesized that the type I and III IFN-induced *Mx1* gene could represent such a restriction factor.

In contrast to most inbred mouse strains such as C57BL/6, B6.A2G mice have an intact *Mx1* gene[12], and the encoded protein Mx1 has a strong antiviral effect against FLUAVs[13,14]. FLUAV NP is an important target of Mx1, and Mx1 has been shown to interact with NP[15]. The NP protein is localized in the nucleus of the infected host cell in the first hours after infection. Since mouse Mx1 also resides in the nucleus and interacts with FLUAV NP, we hypothesize that Mx1 could be a factor that limits the amount of NP antigen and processed NP peptides that can be uploaded in the MHC-I complex. If this is the case, then the mutated rMVA-NP constructs could have an advantage over the rMVA-NPwt construct in the B6.A2G background.

To test our hypothesis, we compared the outcome of a prime-boost vaccination with the different rMVA-NP constructs. The presence of NP-specific $CD8^+$ T cells in the spleen and in cells isolated from the blood were determined post vaccination.

5.2 Results

Groups of five mice (males and females) were vaccinated with rMVA, rMVA-NPwt, rMVA-NPmut, rMVA-NPΔNLS, or rMVA-UbqNP. Immunizations were performed in congenic B6.A2G $Mx1^{-/-}$ and B6.A2G $Mx1^{+/+}$ mice. Mice were vaccinated twice intramuscularly with 10^8 PFU rMVA in the hind legs (50 µl/leg), with a time interval of four weeks. One week after the second immunization, blood and spleens were isolated, and single cells were

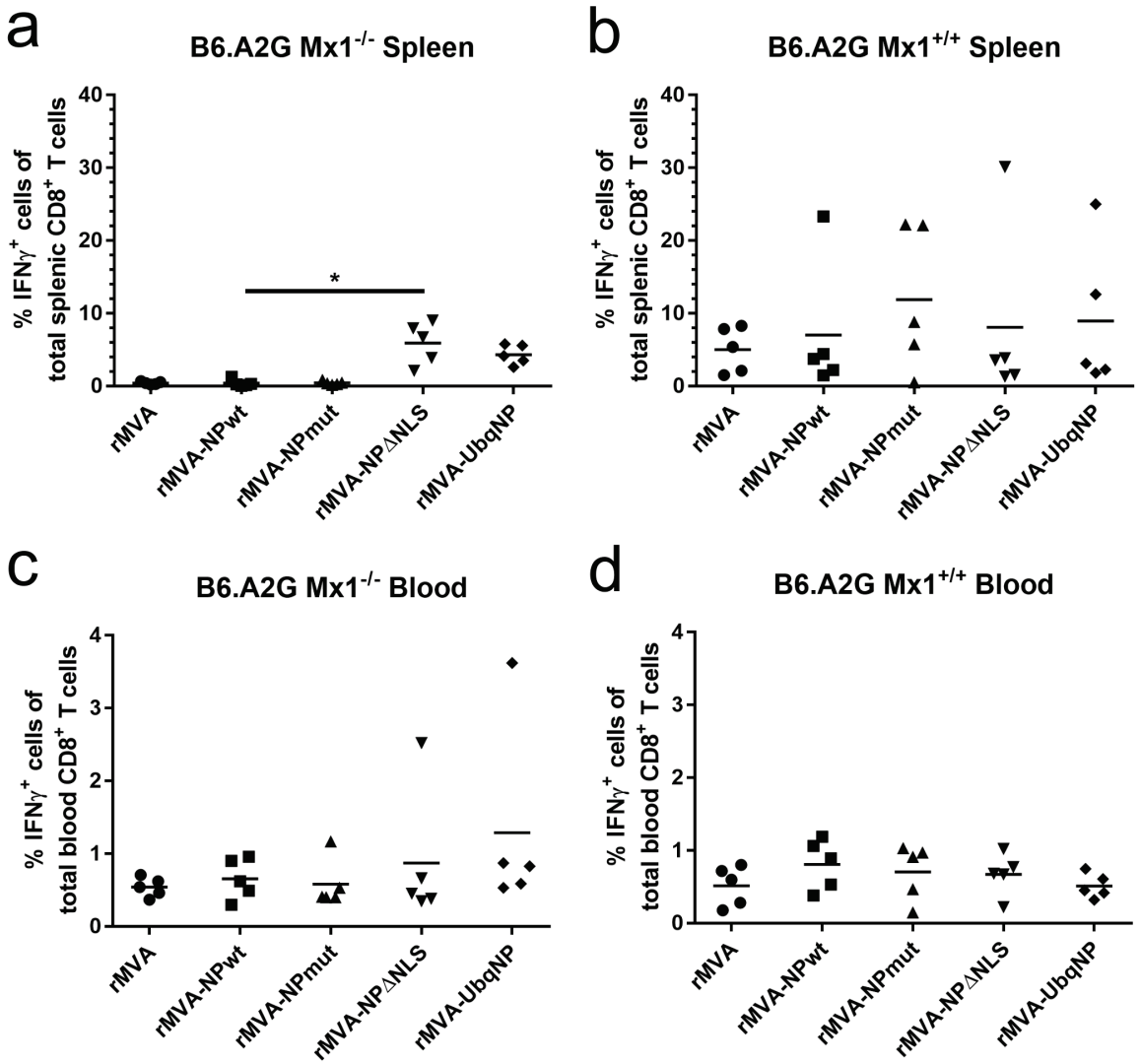

Figure 5.1 ICS analysis of vaccinated B6.A2G Mx1$^{-/-}$ and B6.A2G Mx1$^{+/+}$ mice. B6.A2G Mx1$^{-/-}$ and B6.A2G Mx1$^{+/+}$ mice were vaccinated twice, four weeks apart, with rMVA (negative control) or one of the four indicated rMVA-NP constructs. One week after the second immunization, mice were euthanized, and spleen and blood were isolated. Splenocytes **(a and b)** and PBMCs **(c and d)** were pulsed with the $NP_{366–374}$ peptide. IFNγ-production by CD3$^+$ CD8$^+$ T cells was used as a readout for CD8$^+$ T cell activation. Each data point represents the percentage of CD8$^+$ T cells that are IFNγ$^+$ for a single animal. Data are obtained in one single experiment. Bars indicate means. *, $p < 0.05$, Kruskal-Wallis test with Dunn's multiple comparison test as *post hoc*.

prepared for intracellular cytokine staining (ICS) and enzyme-linked immunospot (ELISPOT) assay. To investigate NP-specific cellular responses, restimulation was done with 4 µg/ml of the H2b-restricted A/PR8-derived $NP_{366-374}$ (ASNENMETM) peptide.

NP-specific CD8$^+$ T cell responses determined in splenocytes of B6.A2G Mx1$^{-/-}$ mice were detectable after vaccination with rMVA-NPΔNLS and rMVA-UbqNP (Figure 5.1a and supplementary figure S5.1). In spleens of B6.A2G Mx1$^{+/+}$ mice we did not detect a significant NP-specific CD8$^+$ T cell response after any of the treatments (Figure 5.1b). Indeed, the high

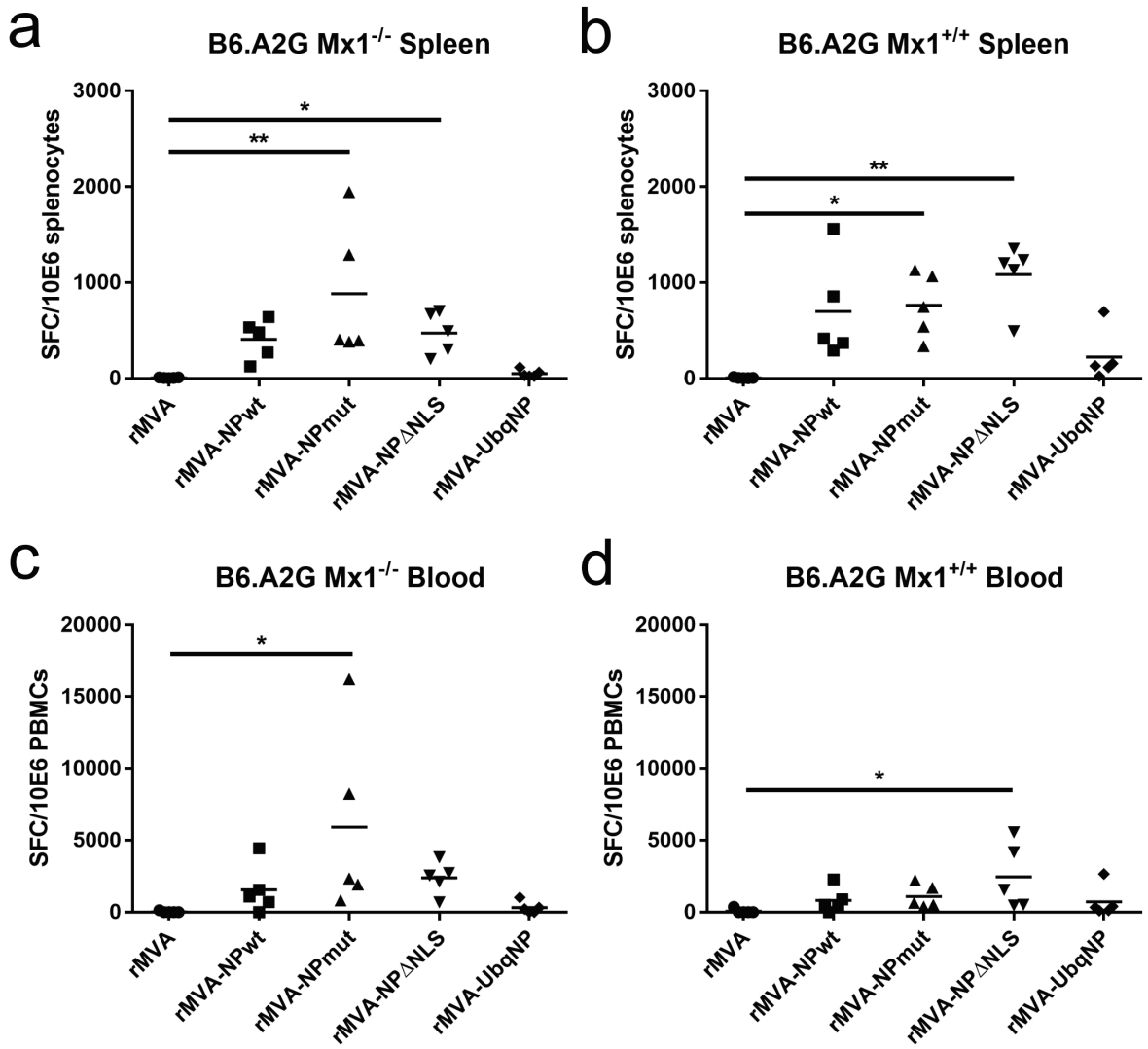

Figure 5.2 ELISPOT analysis of vaccinated B6.A2G Mx1$^{-/-}$ and B6.A2G Mx1$^{+/+}$ mice. B6.A2G Mx1$^{-/-}$ and B6.A2G Mx1$^{+/+}$ mice were vaccinated twice, four weeks apart, with rMVA (negative control) or one of the four indicated rMVA-NP constructs. One week after the second immunization, mice were euthanized, and spleen and blood were isolated. Influenza-specific T lymphocyte responses were measured in splenocytes **(a and b)** and PBMCs **(c and d)** by IFNγ release after stimulation with $NP_{366-374}$ peptide. Each data point represents the amount of IFNγ-producing cells (Spot Forming Cells = SFC) per million splenocytes or PBMCs for a single animal. Data are obtained in one single experiment. Bars indicate means. *, $p < 0.05$; **, $p < 0.01$, Kruskal-Wallis test with Dunn's multiple comparison test as *post hoc*.

NP-responses in splenocytes derived from the negative control vaccinated Mx1$^{+/+}$ mice did not allow to conclude that the MVA-NP vaccination had worked in these mice. ICS analysis of peripheral blood mononuclear cells (PBMCs) did not reveal significant differences between the different treatments, nor between the two mouse strains (Figure 5.1c and d).

ELISPOT analysis of splenocytes revealed that vaccination with rMVA-NPmut and rMVA-NPΔNLS induced a clear and comparable $NP_{366-374}$-specific recall response in B6.A2G Mx1$^{-/-}$ mice that was significantly higher than the responses found in the negative control

vaccinated group (Figure 5.2a). In B6.A2G Mx1$^{+/+}$ mice, we also observed a significant response induced by rMVA-NPmut and rMVA-NPΔNLS, but again no significant differences were observed between these constructs and the wild type NP construct (Figure 5.2b). However, in B6.A2G Mx1$^{+/+}$ mice there does seem to be a trend that the rMVA-NPΔNLS induces a higher induction of $NP_{366-374}$-specific responses. In both mouse strains, based on the ELISPOT analysis, rMVA-UbqNP poorly induces an $NP_{366-374}$-specific T cell response. Finally, in contrast to the ICS data, the ELISPOT assay performed on PBMCs largely reflects the data from the assay on splenocytes (Figure 5.2c and 5.2d).

5.3 Discussion

Our findings from B6.A2G Mx1$^{-/-}$ mice differ from the ones from Altenburg and colleagues in the sense that, based on the ICS assay, we could not detect an NP-specific CD8$^+$ T cell response after vaccination with rMVA-NPwt or rMVA-NPmut. The rMVA-NPΔNLS construct does seem to elicit the strongest antigen-specific CD8$^+$ T cell response, as was also seen in that study. However, these findings are not reflected in the ICS assay performed with PBMCs. This is probably due to the low amount of CD8$^+$ T cells in the blood, and the high variability of the amount of IFNγ$^+$ cells between the different mice. We also noticed elevated background levels for the splenocytes of B6.A2G Mx1$^{+/+}$ mice in the ICS assay (Figure 5.1b and 5.1d). This variation was probably because of heterogeneity in the different treatment groups which were made up of males and females which were bred in our own animal facility.

The ELISPOT assay seems to correlate much better with the results reported by Altenburg *et al.*. This is partly due to the higher sensitivity of this assay in comparison with the ICS assay. The results of our experiments do not support a role for Mx1 in the control of NP-specific T cell responses following vaccination with NP-encoding rMVA vectors. At best, based on the ELISPOT analysis, in B6.A2G Mx1$^{+/+}$ mice there seems to be a trend that the rMVA-NPΔNLS induces a higher induction of $NP_{366-374}$-specific responses (Figure 5.2).

We could not show a clear advantage of the modified rMVA-NP constructs in B6.A2G Mx1$^{+/+}$ mice. The possible role of Mx1 as a factor that limits the amount of NP antigen could thus not be demonstrated. Perhaps intramuscular vaccination with rMVA-NP constructs does not induce a strong enough type I IFN response. However, it has been shown that MVA, in contrast to wild type vaccinia virus, does not produce viral proteins that function as receptor-like inhibitors of type I and type II IFNs[16]. Moreover, infection with MVA can be detected by pathogen-sensing mechanisms in the host, which results in the production of IFNs, cytokines and chemokines[17]. This suggests that *Mx1* expression will likely be induced by the rMVA-NP infected cells *in vivo*, and that mouse Mx1 might not be the limiting factor

for NP antigen presentation after delivery by an MVA vector. Possibly the production of NP is too high for Mx1 to cope with the amount of antigen. Another possibility is that the nuclear phase of the NP protein is too short for mouse Mx1, which resides in the nucleus, to efficiently hinder NP from being processed for antigen presentation.

Future experiments will consist of repeating this vaccination experiment. Additional readouts could be the monitoring of the M1-specific T cell response after vaccination with a plasmid which codes for the FLUAV M1 protein. Since M1 is not targeted by Mx1, it should show us a baseline T cell response in both B6.A2G $Mx1^{-/-}$ and B6.A2G $Mx1^{+/+}$ mice. Another possibility is to measure the specific $CD8^+$ T cell response directed against an alternative epitope such as $NP_{217-225}$. This epitope was shown to be subdominant to the $NP_{366-374}$ epitope[18]. Altenburg *et al.* observed that, *in vitro*, especially the rMVA-NPΔNLS and rMVA-UbqNP constructs could activate low functional avidity NP-specific $CD8^+$ T cell clones more efficiently than the rMVA-NPwt construct. This led to the hypothesis that under suboptimal conditions (when antigen amounts are limited or when $CD8^+$ T cells are of low functional avidity), modifying NP improves the $CD8^+$ T cell response[11]. In our experimental setup and hypothesis, the suboptimal condition is provided by the expression of the Mx1 protein. Conceivably, the effect of Mx1 is more evident on the $CD8^+$ T cell response against a subdominant epitope. In addition, it would also be relevant to test these rMVA vaccines in a mouse strain which expresses the human MxA protein instead of the murine Mx1. This mouse strain expresses a functional human MxA protein in response to IFN exposure in all major organs[19]. In contrast to mouse Mx1, human MxA resides and inhibits FLUAV in the cytoplasm[20]. Since rMVA produces the NP protein in the cytoplasm, MxA can interact with NP at an earlier time point than mouse Mx1. One could argue that MxA could also inhibit the infection by MVA. However, Lorenzo *et al.* recently observed that vaccinia viruses are not susceptible to MxA[21], making this mouse strain a suitable model to further test the rMVA-NP vaccines.

5.4 Materials and methods

Ethics statement. All animal experiments described in this study were conducted according to the national (Belgian Law 14/08/1986 and 22/12/2003, Belgian Royal Decree 06/04/2010) and European legislation (EU Directives 2010/63/EU, 86/609/EEC). All experiments on mice and animal protocols were approved by the ethics committee of Ghent University (permit numbers LA1400091 and EC2017-001).

Mice. Mice were bred in-house under Specific Pathogen Free (SPF) conditions. Mice were housed in individually ventilated cages, in a temperature-controlled environment with 12h light/dark cycles, with food and water *ad libitum*. Congenic B6.A2G $Mx1^{+/+}$ mice carrying a functional A2G *Mx1* allele were kindly provided by Peter Stäheli (University of Freiburg, Germany). Congenic B6.A2G $Mx1^{-/-}$ carrying the defective C57BL/6J *Mx1* allele were generated in our laboratory by crossing B6.A2G $Mx1^{+/+}$ with C57BL/6J ($Mx1^{-/-}$) mice, and subsequent crossing of the heterozygous offspring. For these experiments, littermates were used. Each Group of five mice consisted of a mix of either three males and two females or *vice versa*.

Vaccination. Recombinant MVA constructs were kindly provided by Guus Rimmelzwaan (Department of Viroscience, Erasmus MC, Rotterdam, the Netherlands). Mice (n = 5/group) were vaccinated twice, under mild isoflurane anesthesia, intramuscularly with 10^8 PFU rMVA in the hind legs (50 µl/leg), with a time interval of four weeks.

IFNγ enzyme-linked immunospot assay (ELISPOT). IFNγ enzyme-linked immunospot plates from U-Cytech Biosciences (Utrecht, The Netherlands) were used according to the manufacturer's protocol. Briefly, 96-well immuno-plates were coated with sterile monoclonal anti-IFNγ antibodies and blocked with blocking buffer. One week after the second immunization, the spleens and blood of five mice per group were isolated aseptically and splenocytes/PBMCs were prepared. After lysis of RBCs with NH_4Cl solution, 3×10^5 splenocytes/PBMCs were plated in 100 µl of culture medium supplemented with $NP_{366\text{-}374}$ ASNENMETM peptide at 4 µg/ml. After 16h of peptide restimulation, plates were washed with enzyme-linked immunosorbent assay wash buffer and IFNγ trapped on the plates was detected by a biotinylated polyclonal anti-IFNγ antiserum. GABA-conjugated streptavidin in combination with silver stain reagent resulted in the formation of black silver spots at places where immune cells secreted IFNγ during peptide-restimulation. The spots were counted using an Eli-Expert ELISPOT reader and the Eli.Analyse software (A.EL.VIS GmbH, Hannover, Germany).

Intracellular cytokine staining (ICS) and flow cytometry after *ex vivo* restimulation. One week after the second immunization, spleens and blood of five mice per group were isolated aseptically and splenocytes/PBMCs were prepared. After lysis of RBCs with NH_4Cl solution, 5 x 10^5 splenocytes/PBMCs were plated in 1 ml of culture medium (RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.4 mM Na-pyruvate, nonessential amino acids, 100 U/ml penicillin, and 0.1 mg/ml streptomycin) supplemented with restimulation peptide (described above) at a concentration of 4 µg/ml. After 6h of peptide restimulation, 1 µl Golgiplug (brefeldin A, BD, Erembodegem, Belgium) was added to 1 ml culture medium for measurement of cytokine production by ICS. The Cytofix/Cytoperm kit (BD) was used according to the manufacturer's protocol. Briefly, 10 hours after addition of Golgiplug, cells were stained with a Live/Dead marker (eBioscience Fixable Viability Dye eFluor 450, 65-0866-14, ThermoFisher, 1/1000), and fluorochrome labeled antibodies against CD45 (CD45-AF700, 56-0451-82, ThermoFischer, 1/300), MHCII (MHCII-eFluor450, 48-5321-82, ThermoFischer, 1/500), CD3 (CD3-AF488, 557666, BD Pharmingen, 1/250) and CD8 (CD8-PE-Cy7, 25-0081-81, ThermoFischer, 1/300). Cells were then fixed/permeabilized using the Cytofix/Cytoperm kit (BD), and stained for IFNγ (IFNγ-APC, BD Pharmingen, 554413, 1/100). Cells were then analyzed on an LSR II flow cytometer (BD) with FlowJoX software (Treestar, Ashland, Orlando).

Statistical analysis. Parametric tests did not apply for ICS or ELISPOT samples according to the D'Agostino-Pearson omnibus normality test due to the small number of samples. Therefore, the Kruskal-Wallis test with Dunn's correction was used.

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Supplemental figures

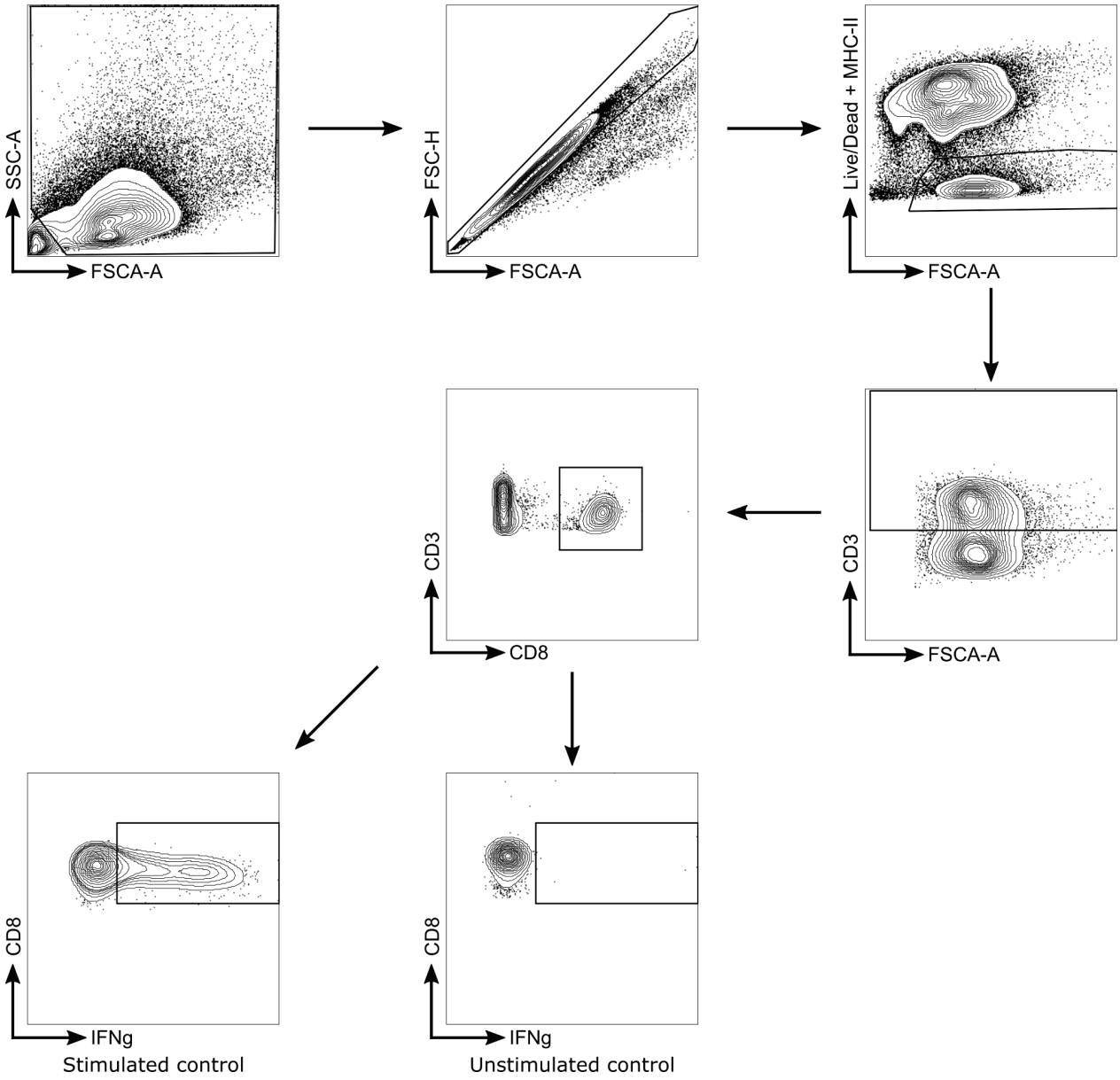

Figure S5.1 Gating strategy of flow cytometry analysis.

PART IV: GENERAL CONCLUSIONS, DISCUSSION AND FUTURE PERSPECTIVES

If we knew what it is we were doing, it would not be called research, would it?

Albert Einstein

The role of Mx1 in the antiviral immune cell compartment

Mx proteins have been elaborately studied during the past 50 years. A lot of data have been produced concerning the innate function and the working mechanism of Mx proteins, mainly for human MxA and murine Mx1 proteins. Our lab also contributed to this very challenging puzzle by demonstrating that mouse Mx1 can interact with FLUAV NP and PB2, and can prevent these proteins from interacting with each other[1]. Moreover, we have shown that mouse Mx1 might actively disrupt existing FLUAV vRNP molecules[2]. However, little is known about the role of Mx1 in the immune cell compartment. This is mainly because most inbred mouse strains used in immunological studies do not express a functional Mx1 protein[3]. The few studies which were performed with $Mx1^{+/+}$ mice concluded that Mx1 has no role in immune cells that confer resistance against FLUAV infection[4-6]. Nonetheless, several other studies provided indirect evidence which shows that certain immune cell types are better protected against a FLUAV infection than others due to an IFN-induced antiviral state[7,8]. The clarification of the role of Mx1 in immune cells after a viral infection was the main issue addressed in this thesis.

The initial goal of this project was to find out if there is a function for the antiviral Mx1 protein in the immune cell compartment. Better said, is Mx1 capable of protecting certain immune cell types which are involved in the immune response against a FLUAV infection? For this, we generated bone marrow chimeras using B6.A2G $Mx1^{-/-}$ mice which do not express a functional Mx1 protein, and B6.A2G $Mx1^{+/+}$ mice which do express a functional Mx1 protein. After a potentially lethal FLUAV infection we did not observe any major effects of Mx1 expression in bone marrow-derived cells. Multiple readouts (body weight, lung viral titers, viral mRNA levels and protein levels) pointed out that the main determinant of resistance is Mx1 expression in the stromal cells. This divergence between $Mx1^{-/-}$ and $Mx1^{+/+}$ recipients was also clear in the levels of eosinophils, moDCs and AMs measured in the lungs after infection. This entails that Mx1 expression in the stromal cells of the recipient mice determines the resistance against or the susceptibility for a lethal FLUAV infection irrespective of Mx1 expression in the bone marrow-derived cell compartment.

Following this, we examined the antigen-specific immune response in bone marrow chimeras after a sublethal FLUAV infection. Ten days after infection we observed the formation of a robust antigen-specific immune response irrespective of the genotype of the bone marrow-derived cells or recipient. This indicated that Mx1 expression in the bone marrow-derived cells does not play a role of significance in the formation of a primary antigen-specific immune response against FLUAV. In addition, this result also suggested that Mx1 expression in stromal cells does not impair the formation of an antigen-specific

immune response against FLUAV. Thus, we can conclude that the expression of a functional Mx1 protein in bone marrow-derived cells is of less importance for resistance against a FLUAV infection than Mx1 expression in the stromal cells, and that Mx1 does not impede the formation of a cellular immune response.

Like Mx proteins, IFITM3 is also the product of an ISG that has been shown to have a strong antiviral function against FLUAV[9,10]. Our results show that FLUAV-infected animals which do not express Mx1 succumb to the consequences of the infection, even when they express high levels of IFITM3. This suggests that IFITM3 alone cannot cope with a possible lethal FLUAV infection. We have shown that expression of Mx1 protein in the stromal cells can protect mice from succumbing to FLUAV infection. To our knowledge, it has never been investigated in an *in vivo* setting whether Mx1 alone, in the absence of IFITM3, can protect animals against a possible lethal FLUAV infection. Thus, it is still possible that Mx1 works together with IFITM3 to confer resistance against FLUAV infections. In this respect, Xiao *et al.* showed an additive antiviral effect when both IFITM3 and human MxA were coexpressed in FLUAV-infected cells[11]. Moreover, it was postulated that MxA cannot fully compensate for the loss of IFITM3 in IFN-treated cells challenged with FLUAV[12]. Interestingly, fusion of MxA with an NLS showed that the working mechanism of MxA is similar to that of murine Mx1 in the nucleus[13,14]. Taking this into account, it is tempting to extrapolate these observations to murine Mx1. However, this cannot be done without caution, because murine Mx1 and human MxA reside in other cellular compartments (nucleus and cytoplasm respectively). This would imply that the antiviral interplay, if any, between IFITM3 and MxA or between IFITM3 and Mx1 could be completely different.

In order to further elucidate the role of Mx1 in immune cells after a viral infection, we implemented a second viral infection model. THOV was chosen because, like FLUAV, it is also an Mx1-sensitive Orthomyxovirus, and small rodents are natural hosts for this virus[15,16]. Bone marrow chimeric mice were infected with a potentially lethal dose of THOV. Unlike the FLUAV infection model, we could readily see an effect of the bone marrow genotype. $Mx1^{-/-}$ recipient mice which received $Mx1^{+/+}$ bone marrow showed less body weight loss, less NP expression, and less pronounced liver pathology than $Mx1^{-/-}$ recipient mice which received $Mx1^{-/-}$ bone marrow. The most plausible reason for this is the THOV tropism for peritoneal cells with a hematopoietic origin[17]. Nonetheless, Mx1 expression in the stromal cells also showed to be the most decisive factor for resistance against THOV infection. Apart from increased morbidity and differential Mx1 and ISG15 production, $Mx1^{-/-} \rightarrow Mx1^{+/+}$ mice showed similar results compared to $Mx1^{+/+} \rightarrow Mx1^{+/+}$ mice after THOV infection. Both groups contained the virus equally well in the sense that the liver viral titers were below the detection limit. In addition, $Mx1^{-/-} \rightarrow Mx1^{+/+}$ and $Mx1^{+/+} \rightarrow Mx1^{+/+}$ mice

showed no liver pathology at microscopic level nor in the serum levels of the enzymes ALT or AST.

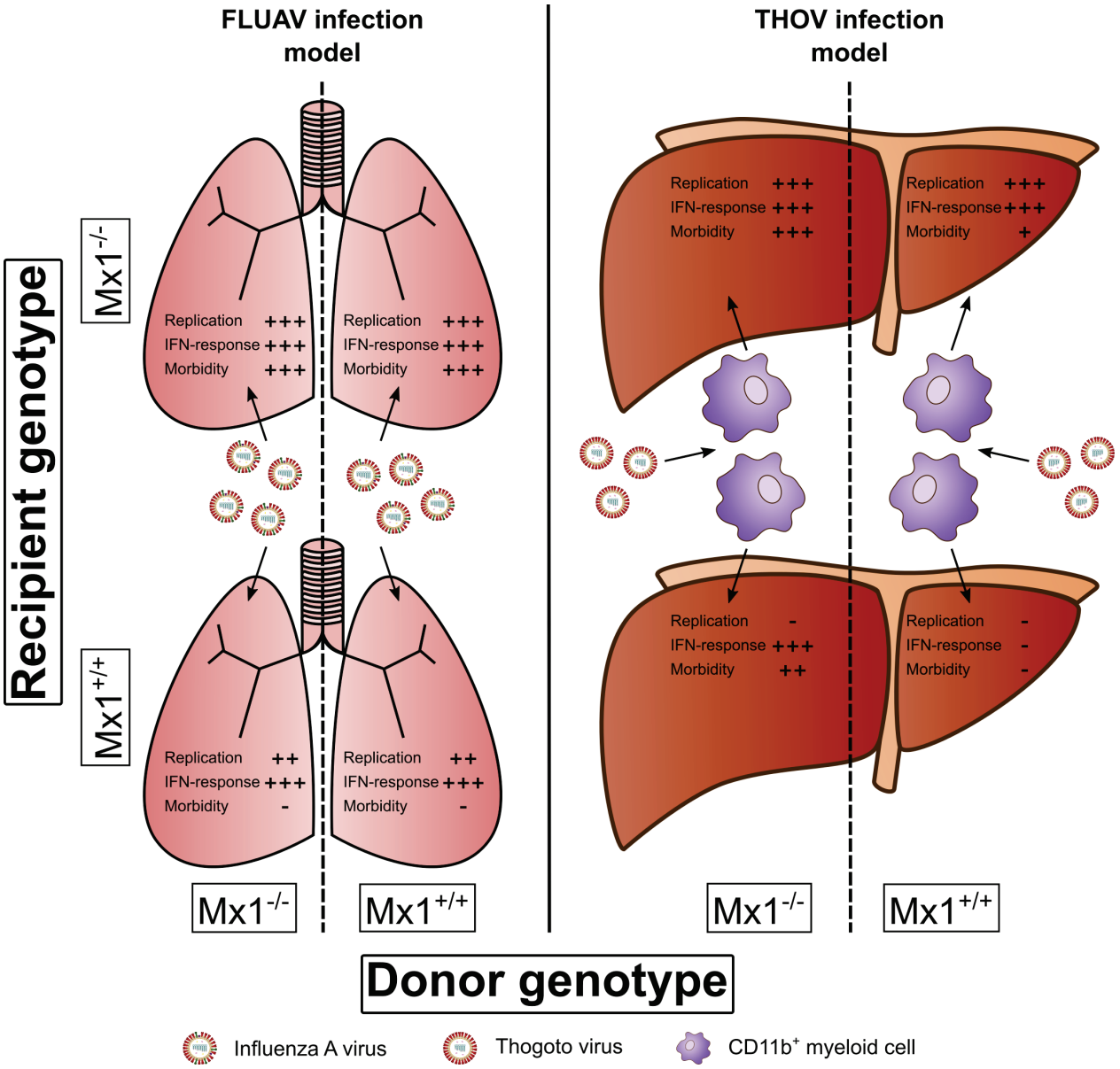

Figure IV.1 The role of Mx1 in the immune cell compartment: summarizing figure. Overview of the results obtained with bone marrow chimeras using two different virus infection models *i.e.* the FLUAV infection model **(left panel)** and the THOV infection model **(right panel)**. For both infection models the studied organ is depicted, and the genotypes of the bone marrow donor and recipient are indicated. Observations concerning replication, type I IFN-response, and morbidity are described for each chimeric group in both models.

In conclusion we can hypothesize that Mx1 can play a significant role in the protection of immune cells, albeit only when the infecting virus has a clear initial tropism for cell types with a hematopoietic origin (Figure IV.1). To completely understand the function of Mx1 in

the resistance against THOV infection, this model should be further explored. For the experiments described in this thesis, the THOV NP and Mx1 expression, along with the influx of immune cells, will be examined in liver sections of the THOV-infected bone marrow chimeras. Viral titers in the blood serum should also be determined. A follow-up bone marrow chimera experiment is being set up wherein other organs – such as spleen, lung, kidney, and brain – will be examined at different time points (0, 1, 2 and 4 dpi) after THOV infection. In this experiment we will also isolate peritoneal lavage fluid, and check the cellular composition at different time points after infection. This experiment will learn us more about the infection kinetics of THOV in different organs. It will be also interesting to see whether or not $Mx1^{+/+}$ bone marrow-derived cells can influence these kinetics. In another bone marrow chimera experiment we will infect the animals with a lower dose of THOV, and follow up the infection beyond four days after infection. This will allow us to see if the $Mx1^{+/+}$ → $Mx1^{-/-}$ and $Mx1^{-/-}$ → $Mx1^{+/+}$ mice will further recover or succumb to the THOV infection. An additional experiment could be the intravenous (instead of intraperitoneal) infection of bone marrow chimeras. By this, the initial barrier imposed by myeloid cells in the peritoneum would be by-passed, leading to a direct infection of hepatocytes. Intravenous infection is also more biologically relevant since THOV is usually transmitted through ticks, which fasten themselves onto the skin and feed on the host's blood. This experiment would further explain the role of a functional Mx1 protein in either the hepatocytes themselves or the peritoneal immune cells.

The THOV infection model led us to hypothesize that Mx1 can play a significant role in the protection of immune cells, albeit only when the infecting virus has a clear initial tropism for cell types with a hematopoietic origin. We will test this hypothesis using influenza viruses. The influenza PR8 strain has been shown to be unable to infect macrophages[18,19]. The main reason for this is the lack of glycosylation sites on the head domain of its HA protein. Other influenza strains which do have glycosylation sites on their HA head domain were able to infect macrophages, albeit not productively. Reading *et al.* showed that, when injected intraperitoneally, influenza PR8 induced a less pronounced local inflammatory response than influenza BJx109 (an influenza virus carrying multiple glycosylation sites on its HA head domain)[19]. Inspired by the work of Reading and colleagues we will generate bone marrow chimeras and infect them intraperitoneally with our maPR8 strain or with another influenza strain which carries glycosylations on its HA head domain. If our hypothesis is correct, then the influenza virus which is able to infect macrophages should be hampered if these macrophages express Mx1. This virus would then give rise to a comparable inflammatory response as is the case for the maPR8 strain.

Triggering the adaptive immune response in B6.A2G mice

Recently, Altenburg *et al.* published their work on the development of recombinant MVA (rMVA) vaccines which express different variants of the FLUAV NP[18]. Their goal was to improve the processing of NP by antigen-presenting cells, which could improve the NP-specific T cells, and they tried this by mutating (rMVA-NPmut) or removing the NLS of NP (rMVA-NPΔNLS), or by amino-terminal fusion of ubiquitin to NP (rMVA-UbqNP). These modifications are meant to enhance the cytosolic retention and degradation of NP. *In vitro*, these constructs indeed improved the activation of NP-specific $CD8^+$ T cell clones in comparison to the rMVA construct which expressed the wild type NP (rMVA-NPwt). *In vivo*, however, vaccination of C57BL/6 mice with these constructs showed no differences between the mutated constructs and the wild type NP construct. They all elicited comparable protection against a lethal influenza A/Puerto Rico/8/34 challenge[18]. As pointed out before, to elicit a strong $CD8^+$ T cell response, the amount of processed antigen should reach a certain threshold. In C57BL/6 mice, the $CD8^+$ T cell response is dominated by T cells specific for the $NP_{366\text{-}374}$ epitope, which can easily reach this threshold. We hypothesized that this is probably the main reason why the modified NP constructs can seemingly not benefit from their enhanced degradation.

Our goal was to test whether the mutated rMVA-NP vaccines have an advantage over the rMVA-NPwt vaccine in B6.A2G $M1^{+/+}$ mice. This mouse strain expresses a functional Mx1 protein, which, based on *in vitro* overexpression experiments, can specifically interact with FLUAV NP, and – because of this – has an antiviral effect against FLUAV[1]. This could be the crucial determinant in limiting the amount of antigen produced by the rMVA-NPwt construct. Since Mx1 resides in the nucleus, the rMVA-NP constructs which have a mutated NLS should already have an advantage over the wild type NP-expressing construct, because the wild type NP derived from the rMVA-NPwt construct has a nuclear phase early after infection[18]. This would give Mx1 the opportunity to interact with this protein and retain it in the nucleus, causing a delay in the antigen processing and presentation, negatively impacting the NP-directed immune response.

To compare the different rMVA constructs, we vaccinated B6.A2G $Mx1^{-/-}$ and B6.A2G $Mx1^{+/+}$ mice twice with the different constructs with a time interval of four weeks. One week after the second vaccination we isolated spleen and blood from these mice, and performed ICS and ELISPOT assays. The ICS assay on both PBMCs and splenocytes showed no obvious differences between the modified constructs and the rMVA-NPwt construct in B6.A2G $Mx1^{+/+}$ animals. The same was true for the ELISPOT assay, although there was a trend that the rMVA-NPmut and especially the rMVA-NPΔNLS constructs show a better $CD8^+$ T cell response than the rMVA-NPwt construct. A possible explanation is that the NP production

by the rMVA vector is too high for Mx1 to handle the produced amount of antigen, or the nuclear phase of NP is too short for Mx1 to have a pronounced effect. Taken together these results do not provide enough evidence to conclude something about the effect of Mx1 on vaccination with the different rMVA constructs. Therefore it is desired to repeat this vaccination experiment. Additional readouts would include the analysis of the M1-specific $CD8^+$ T cell response after vaccination with a FLUAV M1-expressing vector. This could provide us with some kind of baseline T cell response in both B6.A2G genotypes, since Mx1 has not been shown to target the FLUAV M1 protein. Another line of investigation is the examination of the NP-specific $CD8^+$ T cell response directed against an alternative subdominant NP epitope such as $NP_{217\text{-}225}$[19], something which proved instructive in comparing the different MVA-NP constructs *in vitro*[18].

Additionally, it would be interesting to test the different rMVA-NP vaccines in a transgenic mouse strain which expresses human MxA instead of mouse Mx1[20]. MxA also interacts with FLUAV NP, moreover, NP has been shown to be a key determinant of FLUAV sensitivity against Mx1 and MxA[1,21,22]. Since the MxA protein resides in the cytoplasm, it is conceivable that it interacts with NP at an earlier time point than the murine Mx1 protein which resides in the nucleus. Human FLUAV strains which are descendants from the pandemic 1918 H1N1 strain all possess a discrete surface-exposed cluster of three residues – at positions 100, 283, and 313 – which causes these viruses to be more resistant against human MxA and murine Mx1 inhibition[23]. FLUAV NP is also a strong $CD8^+$ T cell antigen and, additional to the resistance against Mx proteins, one could argue that the changed amino acids at positions 100, 283, and 313 could also change certain T cell epitopes. This would have an effect on the efficient induction of $CD8^+$ T cells, like was seen for the $NP_{383\text{-}391}$ epitope when a key residue in this epitope was changed[24]. However, we verified whether the positions in NP which are important for the resistance to Mx1/MxA overlap with human or mouse immunodominant $CD8^+$ T cell epitopes, and this was not the case for all three positions[25].

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Give your heart and soul to me, and life will always be ‘La vie en rose’.

Louis Armstrong

PART V: ADDENDUM

Functional Comparison of Mx1 from Two Different Mouse Species Reveals the Involvement of Loop L4 in the Antiviral Activity against Influenza A Viruses

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Contribution of Jan Spitaels:

Comparison of the antiviral activity of newly isolated SMx1 cDNA (SMx1) to the one described previously in a PR8 minireplicon assay (Figure 2). Comparison of the activities of different SMx1 and A2G Mx1 constructs in a minireplicon assay derived from PR8 or maSwO (Figure 8).

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Functional Comparison of Mx1 from Two Different Mouse Species Reveals the Involvement of Loop L4 in the Antiviral Activity against Influenza A Viruses

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ABSTRACT

The interferon-induced *Mx1* gene is an important part of the mammalian defense against influenza viruses. *Mus musculus* Mx1 inhibits influenza A virus replication and transcription by suppressing the polymerase activity of viral ribonucleoproteins (vRNPs). Here, we compared the anti-influenza virus activity of Mx1 from *Mus musculus* A2G with that of its ortholog from *Mus spretus*. We found that the antiviral activity of *M. spretus* Mx1 was less potent than that of *M. musculus* Mx1. Comparison of the *M. musculus* Mx1 sequence with the *M. spretus* Mx1 sequence revealed 25 amino acid differences, over half of which were present in the GTPase domain and 2 of which were present in loop L4. However, the *in vitro* GTPase activity of Mx1 from the two mouse species was similar. Replacement of one of the residues in loop L4 in *M. spretus* Mx1 by the corresponding residue of A2G Mx1 increased its antiviral activity. We also show that deletion of loop L4 prevented the binding of Mx1 to influenza A virus nucleoprotein and, hence, abolished the antiviral activity of mouse Mx1. These results indicate that loop L4 of mouse Mx1 is a determinant of antiviral activity. Our findings suggest that Mx proteins from different mammals use a common mechanism to inhibit influenza A viruses.

IMPORTANCE

Mx proteins are evolutionarily conserved in vertebrates and inhibit a wide range of viruses. Still, the exact details of their antiviral mechanisms remain largely unknown. Functional comparison of the *Mx* genes from two species that diverged relatively recently in evolution can provide novel insights into these mechanisms. We show that both *Mus musculus* A2G Mx1 and *Mus spretus* Mx1 target the influenza virus nucleoprotein. We also found that loop L4 in mouse Mx1 is crucial for its antiviral activity, as was recently reported for primate MxA. This indicates that human and mouse Mx proteins, which have diverged by 75 million years of evolution, recognize and inhibit influenza A viruses by a common mechanism.

The Mx proteins are interferon (IFN)-induced GTPases that inhibit a wide range of viruses, including *Orthomyxoviridae*, *Bunyaviridae*, and *Rhabdoviridae* (reviewed in references 1 and 2). The gene encoding mouse Mx1, the founder member of this family of antiviral proteins, was discovered almost 30 years ago on the basis of the resistance of the A2G mouse strain to influenza A virus infection (3, 4). This resistance is inherited as a dominant autosomal trait and depends on a single gene (*Mx1*) located on chromosome 16 (5). However, most inbred laboratory mouse strains contain a three-exon deletion or a nonsense mutation in the *Mx1* locus and are susceptible to influenza viruses (6). In contrast, $Mx1^+$ and $Mx1^-$ alleles can be found at similar frequencies in wild mice. This suggests that there is a selective advantage of heterozygosity at the *Mx1* locus, as one would expect that the $Mx1^+$ allele would otherwise be fixed in wild mouse strains (7). The mouse *Mx* locus contains *Mx1* and *Mx2*. Remarkably, *Mx2* is also nonfunctional in laboratory mouse strains but functional in wild mouse strains (8, 9). It is unclear why laboratory mouse strains lack functional *Mx* genes. One possibility is a founder effect, as most laboratory strains are derived from a small number of mice. Other possibilities are the absence of positive selection for a functional *Mx* locus or a selective advantage for an Mx^- locus in laboratory mice (6, 7).

Mx1 expression is induced by type I and type III interferons and can protect mice against influenza A virus infection (10–13). However, Mx1 can protect cells against influenza A virus infection in the absence of interferons (14, 15). The molecular details of the anti-influenza virus mechanism of mouse Mx1 are only partially resolved. There is strong evidence that Mx1 inhibits the activity of the viral polymerase, which is present in viral ribonucleoproteins (vRNPs) (16–18). These vRNPs are the minimal units required for viral transcription and replication. They contain the viral RNA (vRNA) genome complexed with multiple nucleoprotein (NP) molecules and one RNA-dependent RNA polymerase complex containing polymerase basic protein 1 (PB1), PB2, and polymerase acid protein (PA) (19). We recently showed that Mx1 interacts

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Chemical-controlled Activation of Antiviral Myxovirus Resistance Protein 1

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Chemical-controlled Activation of Antiviral Myxovirus Resistance Protein 1*

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The antiviral myxovirus resistance protein 1 (MX1) is an interferon-induced GTPase that plays an important role in the defense of mammalian cells against influenza A viruses. Mouse MX1 interacts with the influenza ribonucleoprotein complexes (vRNPs) and can prevent the interaction between polymerase basic 2 (PB2) and the nucleoprotein (NP) of influenza A viruses. However, it is unclear whether mouse MX1 disrupts the PB2-NP interaction in the context of pre-existing vRNPs or prevents the assembly of new vRNP components. Here, we describe a conditionally active mouse MX1 variant that only exerts antiviral activity in the presence of a small molecule drug. Once activated, this MX1 construct phenocopies the antiviral and NP binding activity of wild type MX1. The interaction between PB2 and NP is disrupted within minutes after the addition of the small molecule activator. These findings support a model in which mouse MX1 interacts with the incoming influenza A vRNPs and inhibits their activity by disrupting the PB2-NP interaction.

The myxovirus resistance (*MX*) genes are evolutionarily conserved in nearly all vertebrates. *MX* gene expression is induced by type I or III interferon, and the corresponding gene products can inhibit a wide range of viruses (1). Human MxA, for example, can suppress the replication of influenza and Thogoto viruses (both Orthomyxoviridae), vesicular stomatitis virus (a rhabdovirus), and hepatitis B virus (a hepadnavirus), and mouse MX1 inhibits influenza and Thogoto virus replication (2).

MX proteins are classified as large GTPases (3, 4). The crystal structure of MxA revealed how the GTPase domain, the bundle-signaling element (BSE),[4] and the stalk domain are positioned relative to each other in space (5). These three domains each have specific functions in antiviral activity. The GTPase domain is the most conserved part in the family of large GTPases, and the capacity of MX to bind with GTP determines its antiviral activity (6). The BSE is connected to the GTPase domain via a hinge. Gao *et al.* (5, 7) suggested that this BSE is crucial for transmitting conformational changes, caused by GTPase activity, to the third domain of MX, *i.e.* the stalk. The stalk domain is important for oligomerization and target recognition. It contains three interfaces and a loop region (loop L4), which mediate oligomerization through a crisscross interaction pattern. This ultimately results in the formation of oligomeric rings with the stalk domains pointing inward and the GTPase domains located at the periphery of the ring. Loop L4, present at the tip of this stalk, and directed toward the center of the MxA oligomeric ring, is important for viral target recognition (8–10). A yet unproven model proposes that MX proteins, organized in rings, wrap around their viral targets (*e.g.* the vRNPs) and cooperatively inhibit or disturb the function of those viral targets. However, this model has recently been challenged by the results of Nigg and Pavlovic (11), who reported that oligomerization is not crucial for the antiviral activity of human MxA.

Substantial progress has been made in the last few years in our understanding of the molecular details of the antiviral mechanism of MX proteins. However, it remains unclear how MX proteins interact with influenza A vRNPs and what the molecular consequences are of such an interaction. There is clear evidence that human influenza A viruses are more resistant to human MxA than avian influenza viruses are (12). This difference in sensitivity is associated with amino acid differences in the nucleoprotein (NP) of human and avian influenza A viruses (13–15). This suggests that NP is a direct or indirect target of mammalian MX1 proteins. In line with this, we and others previously showed that mouse MX1 can interact with NP. There is also evidence that influenza A PB2 is a target of and binds with mouse MX1 (14, 16, 17).

PB2 and NP are part of the vRNPs, which are the minimal units required for influenza RNA transcription and replication. The vRNPs consist of the viral RNA genome, multiple NP molecules, and one RNA-dependent RNA polymerase complex containing PB1, PB2, and polymerase acidic protein (PA) (18). We showed that the interaction between NP and PB2 is strongly reduced in the presence of MX1 (10, 14, 19). An appealing model is therefore that mouse MX1 prevents or dis-

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[1] Both authors contributed equally to this work.

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[4] The abbreviations used are: BSE, bundle-signaling element; NP, nucleoprotein; RNP, ribonucleoprotein; vRNP, viral RNP; PA, polymerase acidic protein; FKBP, FK506 binding protein; FRB, FKBP rapamycin-binding protein; NLS, nuclear localization signal; MOI, multiplicity of infection; CDTA, 1,2-cyclohexylenedinitrilotetraacetic acid; NEM, *N*-ethylmaleimide.

Potent Single-Domain Antibodies that Arrest Respiratory Syncytial Virus Fusion Protein in its Prefusion State

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Contribution of Jan Spitaels:

Analysis of the immune cell influx in the lungs by flow cytometry on bronchoalveolar lavage fluid (Figure 6).

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Potent single-domain antibodies that arrest respiratory syncytial virus fusion protein in its prefusion state

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Human respiratory syncytial virus (RSV) is the main cause of lower respiratory tract infections in young children. The RSV fusion protein (F) is highly conserved and is the only viral membrane protein that is essential for infection. The prefusion conformation of RSV F is considered the most relevant target for antiviral strategies because it is the fusion-competent form of the protein and the primary target of neutralizing activity present in human serum. Here, we describe two llama-derived single-domain antibodies (VHHs) that have potent RSV-neutralizing activity and bind selectively to prefusion RSV F with picomolar affinity. Crystal structures of these VHHs in complex with prefusion F show that they recognize a conserved cavity formed by two F protomers. In addition, the VHHs prevent RSV replication and lung infiltration of inflammatory monocytes and T cells in RSV-challenged mice. These prefusion F-specific VHHs represent promising antiviral agents against RSV.

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Curriculum Vitae

Personal information

Name	Jan Spitaels
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Date of Birth	September 5, 1990
Place of Birth	Dendermonde
Nationality	Belgian

Education

2014-2018 **PhD in Biochemistry and Biotechnology**
Doctoral thesis: Determining the contribution of Mx1 to the antiviral immune cell compartment
VIB – UGent Center for Medical Biotechnology, Ghent, Belgium
Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium

2011-2013 **Master of Science in Biochemistry and Biotechnology**
Major option: Microbial Biotechnology
Minor option: Biochemistry and Structural Biology
Master Thesis: Structuur-functie analyse van HRSV F-neutraliserende nanobodies
Ghent University, Ghent

2008-2011 **Bachelor of Science in Biochemistry and Biotechnology**
Ghent University, Ghent

2002-2008 **Science-Mathematics**
Heilige-Maagd College, Dendermonde

Languages

- **Dutch:** native language
- **English:** very good
- **French:** good working knowledge

Informatics

- Microsoft Office (Word, Excel, Powerpoint, Outlook), Graphpad Prism software, FlowJo X software, and qbase+ software

Courses and trainings followed

2016 How To Design, Perform, & Publish 10-18-Color Flow Cytometry Experiments
September 22, ExCyte, Ghent, Belgium

2015 Internship at Icahn School of Medicine at Mount Sinai
García-Sastre Laboratory
Guiding person: Michael Schotsaert
July 4 – August 6, New York City, USA

2014 Two Day Flow Cytometry Boot Camp
October 2 – 3, ExCyte, Leuven, Belgium

Basiscursus Flowcytometrie
May 14, BD, Erembodegem, Belgium

Certificate laboratory animal science FELASA category B and C
January, Ghent University, Ghent, Belgium

Publications in Scientific Journals

Verhelst J., Spitaels J., Nurnberger C., De Vlieger D., Ysenbaert T., Staeheli P., Fiers W., Saelens X.
Functional comparison of Mx1 from two different mouse species reveals the involvement of loop L4 in the antiviral activity against influenza A viruses
Journal of Virology 89, 10879-10890, 2015. (Epub 2015 Aug 19)

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Influenza and Memory T Cells: How to Awake the Force
Vaccines 4(4), 33, 2016 (Epub 2016 Oct 13)

Verhelst J., Van Hoecke L., Spitaels J., De Vlieger D., Kolpe A., Saelens X.
Chemical-controlled activation of antiviral Myxovirus Resistance 1
Journal of Biological Chemistry 292, 2226-2236, 2017 (Epub 2016 Dec 23)

Rossey I., Gilman M.S.A., Kabeche S.C., Sedeyn K., Wrapp D., Kanekiyo M., Chen M., Mas V., Spitaels J., Melero J.A., Graham B.S., Schepens B., McLellan J.S. & Saelens X.
Potent single-domain antibodies that arrest respiratory syncytial virus fusion protein in its prefusion state
Nature Communications 8, 14158, 2017 (Epub 2017 Feb 13)

Teaching Activities

2016	Guidance Master I project (Rok Razpotnik) 'Functional analysis of labeled anti-mouse Mx1 polyclonal antibody'
Others	Wetenschap In De Kijker (2014)

Participation (Inter)National Conferences

Oral Presentations

Fifth annual Belvir meeting (Brussels, belgium)
December 7 2017

Virus – IFN meeting (Brussels, Belgium)
June 13 2017

Fifth International Influenza Meeting (Münster, Germany)
September 26 2016

MBC Lunch Seminar (Ghent, Belgium)
May 6 2015

Others

Fourth annual Belvir Meeting
December 8 2016, Brussels, Belgium

Third annual Belvir Meeting
December 18 2015, Brussels, Belgium

VIB Seminar 2015
March 30 – April 1 2015, Blankenberge, Belgium

Second annual Belvir Meeting
December 8 2014, Brussels, Belgium

Fourth International Influenza Meeting
September 21-23 2014, Münster, Germany

IRC Predoc Symposium
June 20 2014, Ghent, Belgium

VIB Seminar 2014
April 28-30 2014, Blankenberge, Belgium

Dankwoord

We zijn er. Vier jaar en elf dagen na de start ben ik aan 'den arrivé' van mijn doctoraat beland. Hoewel veel mensen denken dat dit een zeer individueel parcours is geweest, is er toch een hele waslijst aan mensen die ik moet bedanken. Het liefst van al zou ik al deze mensen eens goed tegen mijne gilet willen trekken en drie (of meer) kussen geven, voornamelijk op de wangen al kan ik andere lichaamsdelen niet uitsluiten. Het is mij echter duidelijk gemaakt dat dit 'not done' is, dus zal ik het bij de klassieke bedankingen houden. Ik wil mij bij deze op voorhand excuseren indien ik iemand vergeet te bedanken. Het ligt niet aan u, maar eerder aan de steeds talrijker wordende gaten in mijn geheugen.

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aan jou gehad tijdens het schrijven. Al was het maar een korte babbel over 'Wa zijde gij nu aant schrijven?'. Veel succes met je publieke verdediging, en met je nieuwe project! **Dorien**, ik heb nog niet veel mensen zich met zoveel vastberadenheid weten vastbijten in hun doctoraat. Je vastberadenheid, competitieve geest en je mentaliteit om nooit op te geven, zullen er voor zorgen dat je een dijk van een doctoraat zal hebben. Nog een jaar vollen bak gaan, en dan is het uw beurt om af te leggen. **Anna**, in a couple of months it will be you turn to defend your PhD thesis. Good luck with finalizing the work and writing your thesis! **Marlies**, nog geen jaar in het labo en je bent al een begrip voor iedereen. Moest er een schaal bestaan voor 'hard werken' dan zou 'Marlies' de SI-eenheid zijn. Merci voor de hulp bij mijn eerste stapjes in de histologie! **Jackeline**, je bent nog niet zolang in het labo, dus ik ken je nog niet zo goed. Ik denk wel dat ik voor iedereen spreek, als ik zeg dat je echt een aanwinst bent voor het labo. We misten nog wat Zuid-Amerikaanse furie. **Tine**, in bouwvakkertermen zou men je een draagmuur noemen. Als jij wegvalt staat heel het labo op losse schroeven. Bedankt om mij steeds goedgeluimd bij te staan als ik vragen had over *in vivo* experimenten, administratie, pakketjes versturen, en zoveel meer! **Anouk**, de moederkloek van het labo. Ik weet dat je dat niet zo graag hoort, maar ik kon het niet laten. Voor iemand die eigenlijk nogal timide is, kan je in je enthousiasme toch soms veel decibels produceren. ☺ Merci om mij steeds te helpen bij kloneringen, DNA opzuiveringen, producties van virussen, en vooral om gewoon te luisteren als er iets scheelde. **Ioanna**, soon you will also be defending your PhD. Good luck, and all the best for you and your family! **Emma**, thanks for always helping me with good advice. Your expertise in the macrophage area was much appreciated. Good luck with keeping up the Aussie-vibe in the lab!

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The future is a gift from tomorrow.
Author unknown